Systemic sclerosis: are anti-nuclear antibodies our guiding stars?

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General introduction

Systemic Sclerosis – from mild to life-threatening condition

Systemic Sclerosis (SSc) is a connective-tissue disease that is characterized by vasculopathy, auto-immune phenomena and fibrosis in a wide range of organs. With a prevalence estimated between 150-443 per million and an incidence between 10-20 patients, per million per year (1, 2), the disease is classified as a rare disease.

Based on the extent of skin involvement, the disease is classified in three subtypes: non-cutaneous, limited cutaneous (lcSSc) and diffuses cutaneous SSc (dcSSc). In non-cutaneous SSc the skin is not involved, in lcSSc skin involvement is limited to the parts distal from elbows and knees and may involve the face, while in dcSSc also skin of more proximal parts of the body is involved, including the upper arms, upper legs and/or trunk (Figure 1) (3).

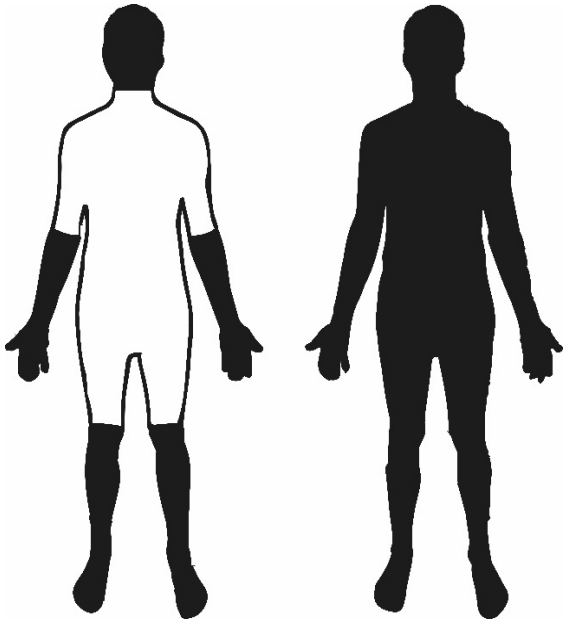

Figure 1. Limited (left) and diffuse (right) cutaneous Systemic Sclerosis

Apart from these distinct subtypes, the symptoms patients may experience can vary from 'only' Raynaud's phenomenon and sclerodactyly, to diffuse cutaneous involvement with cardiac rhythm disturbances and severe dyspnoea caused by heart and lung involvement. This heterogeneous presentation occurs throughout the disease course. Some patients have live-long mild disease, with only minor complications interfering with daily life, while others die from severe organ complications shortly after disease onset.

Complications that may occur during the disease include pulmonary arterial hypertension (PAH), interstitial lung disease (ILD), cardiomyopathy and renal crisis. These complications affect the life-expectancy significantly, resulting in a standardized mortality ratio (SMR) of 3.5, which has not changed over the past 40 years (4).

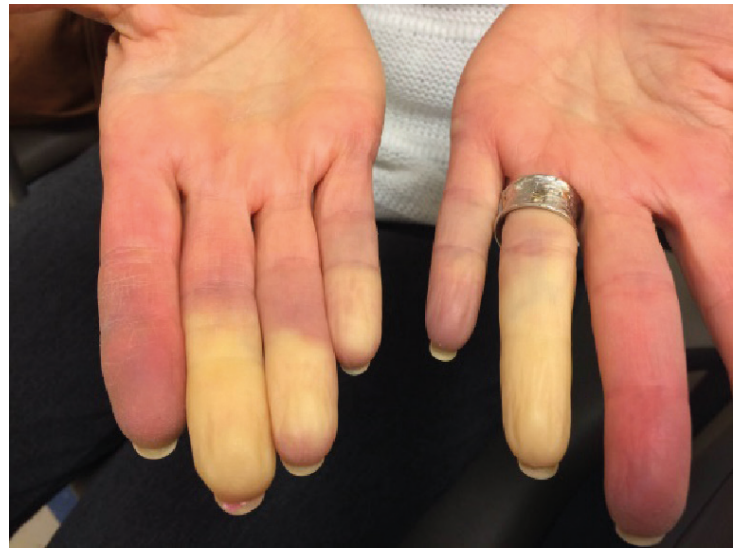

Figure 2. Raynaud's phenomenon –episodes of vasoconstriction of area's in the fingers, as a reaction to cold and/or emotion. Parts involved turn white and may turn blue and with return of blood flow red discoloration with burning sensation can occur.

History

In 1945 it was Robert Goetz, who introduced the term 'Systemic Sclerosis', as we nowadays use it in clinical practice and throughout this thesis. Cases compatible with the disease have been described however long before. Already in 1731, Carlo Curzio described a case-report that may have represented the disease (5). Curzio described a case of a 17-year-old woman with excessive tension and hardness of her skin over all her body, by which she was so restricted that she could hardly move her limbs. In that time, the treatment of the girl consisted of warm milk and vapor baths, bleeding from the foot and small doses of quicksilver. After 11 months of treatment her skin was described to be "perfectly soft and flexible" again. Later, also other manifestations of the disease were observed by various physicians. In 1847, Forget described involvement of many joints. In 1878, Weber noted the coexistence of calcinosis with the disease. In 1865, Raynaud noted that the disease started with vasomotor changes in the fingers, we now call Raynaud's phenomenon (Figure 2). Notably, for a long time, symptoms of the lung and gastro-intestinal tract were considered a consequence of skin fibrosis (due to lack of room to expand), rather than the result of direct involvement of lung and gut involvement. In 1898, a pathological

examination of the lungs by Notthaft provided new insights, as he discovered that pulmonary blood vessels were found to be enveloped in a concentric connective tissue shell, with the media of the arteries was markedly thickened and the media and intima containing cellular infiltrate, the latter being markedly proliferated. These progressive insights led by the conclusion of several researchers later in time of what thus far was called scleroderma, actually being a systemic disease (5).

Pathogenesis

Until today, the disease pathogenesis of SSc is not fully understood. Historic hypotheses include SSc being a result of nervous system dysfunction (6) or thyroid dysfunction (7). These are not today's prevailing views. Currently, three major contributors are recognized in the etiology of SSc: I) microangiopathy; II) excessive fibrosis and III) dysregulated immunity (8). Under the influence of environmental, genetic and stochastic factors, these three factors have an interplay resulting in the disease in all its forms. In the following paragraphs, these three factors will be discussed in more detail.

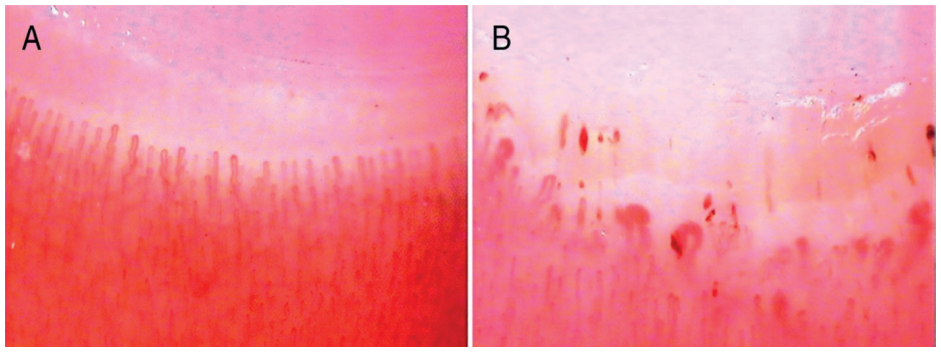

Figure 3. Nailfold microscopy images – left: a normally constructed nailbed, right: the nailbed of an SSc patient with less capillaries, enlarged capillaries and bleedings.

Microangiopathy might be the first event in disease pathogenesis (9). This is further supported by the fact that Raynaud's phenomenon often precedes clinically recognizable SSc. The typical SSc microangiopathy can be observed in this preclinical phase by nailfold microscopy (10). In 1973, Maricq and LeRoy were the first to describe that capillaries in the nailfold bed of patients exhibit bleedings, loss and enlargement (Figure 3) (11). Histopathologic understanding of these changes followed in the years after. In 1980, Fleischmajer and Perlish described that the earliest vascular changes in patients were the opening of tight junctions between endothelial cells, vacuolization of the cytoplasm with an increase in the number of basal lamina-like layers and occasional entrapment of lymphocytes and vesicles in the vessel walls (12). In the same year Rodnan et al. showed that microvessels of patients with longstanding disease showed severe intimal thickening and adventitial fibrosis (13). Till date, the underlying origin of these vascular changes is still unclear. A recent hypothesis is

that microangiopathy in SSc is the result of defects in vasculogenesis (14-18). Herein, abnormalities in bone-marrow derived endothelial progenitor cells may account for the vascular disease, but the precise mechanism remains unknown.

Excessive fibrosis is the result of imbalance in the regulation of the extracellular matrix (ECM). In early phases of SSc skin histology shows edema and perivascular inflammatory infiltrates with lymphocytes and monocytes in the papillary and reticular dermis. In later stages a prominent accumulation of ECM, together with obliteration and loss of vessels and skin appendages is observed (12). In parallel to the skin, these changes also occur in other organs. For instance in the lung, early disease is histopathologically characterized by interstitial edema, intermediate disease by obliteration of terminal air spaces by fibrous connective tissue and in late disease these obliterations are characterized by scar tissue and microcysts (19). Normally, the amount of ECM is regulated by two processes: 1) the release of collagen from activated fibroblasts and 2) degradation of the ECM by matrix metalloproteinase and other matrix-degrading enzymes (20). Myofibroblasts are a specific type of fibroblast expressing α-smooth muscle actin, with a chronically activated phenotype. They are known to be critical in wound healing (20-22). In SSc, the number of myofibroblasts present in the skin is associated with the clinical skin score (23). Therefore, these myofibroblasts seem to play an important role in SSc pathogenesis. Formation of these myofibroblasts is largely driven by TGF-β, but additionally, other mechanisms and chemokines are needed to result in the typical fibrosis (24, 25). Morphogen pathways like Wnt-, Hedgehog and Notch-signalling cascades are shown to be activated in SSc. Whether they are in fact the drivers of fibrotic complications in SSc remains to be elucidated (26).

Last, there are several observations that point to the immune system being part of disease pathogenesis. For example, in early skin lesions infiltration of oligoclonal T cells is observed (27). Also, improvement after immunosuppressive therapies such as autologous hematopoietic stem cell transplantation (28, 29), cyclophosphamide (30-34) and rituximab (35) point at a role of the immune system in the disease pathogenesis. Moreover, there is the presence of disease-specific autoantibodies (36-38), which role is the main subject of this thesis. Over 95% of SSc patients have anti-nuclear autoantibodies (ANAs). Anti-topoisomerase I (ATA) and anti-centromere antibodies (ACA) are the most common specific auto-antibodies in SSc (39, 40). They occur in respectively 20-30% and 30-40% of patients. Additionally, at least five other SSc specific auto-antibodies have been described. All these antibodies are associated with disease specific features (Table 1). Their direct role in pathogenesis is not clear. Unravelling the exact link between auto-immunity on the one hand and fibrosis and vasculopathy on the other hand, might be key to the disease pathogenesis.

Table 1. Autoantibodies in Systemic Sclerosis and their main clinical associations

Autoantibody	Frequency	Clinical associations
Anti-centromere (ACA)	16-39%	lcSSc; PAH without ILD; PBC; protective for ILD and scleroderma renal crisis
Anti-topoisomerase I (ATA)	9-39%	dcSSc>lcSSc; ILD; severe digital vasculopathy
Anti-RNA polymerase III (RNApIII)	4-25%	dcSSc; scleroderma renal crisis
Anti-Th/To (ThTo)	1-7%	lcSSc; ILD; PAH
Anti-fibrillarin (U3RNP)	1-6%	dcSSc>lcSSc; severe disease; muscle involvement; PAH
Anti-Pm-Scl (PmScl)	0-6%	polymyositis/dermatomyositis overlap, arthritis overlap; ILD
Anti-Ku (Ku)	1-3%	muscle and joint involvement
Anti-U1RNP (U1RNP)	5-35%	overlap syndromes

dcSSc-diffuse cutaneous systemic sclerosis, ILD-interstitial lung disease, lcSSc-limited cutaneous systemic sclerosis, PAH-pulmonary arterial hypertension, PBC-primary biliary cirrhosis,

* Table derived from Nihtyanova and Denton, 2010 (41)

Diagnosis and classification

The diagnosis of SSc is primarily based on clinical symptoms and observations. To enable clinical trials with homogeneous patient selection, the American College of Rheumatology (ACR) developed classification criteria in 1980 (ACR 1980 SSc classification criteria; Table 2)(42). In collaboration with the European League Against Rheumatology (EULAR) the current ACR/EULAR 2013 classification criteria for SSc (Table 3) have been developed (43).

The main difference between the ACR 1980 criteria and the ACR/EULAR 2013 criteria is the capability of the latter to include patients that have limited disease and patients at an early stage. This is highly important for clinical research, as conclusions depend on the clinical phenotype and disease duration of the patients included.

Table 2. ACR 1980 preliminary classification criteria for Systemic Sclerosis

	disease feature	Definition
Major criterium	proximal scleroderma	sclerodermatous involvement proximal to the digits, affecting proximal portions of the extremities (i.e., forearms, arms, legs, thighs, and always including the digits as well), the face, neck or trunk.
Minor criteria	sclerodactyly	tightness, thickening and no-pitting induration, limited to fingers and toes
	digital pitting scars of fingertips or loss of substance of the distal finger pad	depressed areas at tips of digits or loss of digital pad tissue as a result of digital ischemia rather than trauma or exogenous causes
	bibasilar pulmonary fibrosis	bilateral reticular pattern of linear or lineonodular densities which are most pronounced in basilar portions of the lungs in standard chest roentgenogram; may assume appearance of diffuse mottling or “honeycomb lung” and should not be attributable to primary lung disease

A patient meets the ACR 1980 criteria when either fulfilling the major criterium or ≥2 minor criteria

For many rheumatic diseases, a window-of-opportunity has been suggested (44-46). This hypothesis indicates a period very early in disease course where targeted interventions can interfere with progression to full-blown disease and prevent severe disease complications or even interfere with disease development. Under this hypothesis, cohorts like the ‘Clinical Suspect Arthralgia’ (CSA) for rheumatoid arthritis (RA) (47) and SPACE for spondylarthritis (SPA) (48) in Leiden, but also the Very Early Diagnosis Of Systemic Sclerosis (VEDOSS) have originated (49).

Initiatives of early identification of SSc have resulted in the knowledge that microangiopathy, but also disease specific auto-antibodies are present in SSc in a preclinical phase, in which the patient only has complaints of Raynaud’s phenomenon (10, 50-52). From studies in very early SSc we know that in patients with Raynaud’s, the finding of either an SSc-specific antibody or specific nailfold capillary changes (dilatations >30um, avascular areas or capillary loss) results in a chance of approximately 1/3 of developing SSc in the near future. Finding these two features together results in a chance of ~75% of developing SSc.

Table 3 | The ACR/EULAR 2013 classification criteria for systemic sclerosis

Item	subitem	Definition	weight/ score
skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints *(sufficient criterion)*	-	Skin thickening= thickening or hardening not due to scarring after injury, trauma, etc	9
skin thickening of the fingers *(only count the higher score)*	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 of the digital bone and joint structures. Swelling of the digits obliterates these contours. Not due to other causes such as inflammatory dactylitis.	2
	sclerodactyly of the fingers	Skin thickening or hardening distal to the metacarpophalangeal joints but proximal to the proximal interphalangeal joints	4
fingertip lesions *(only count the higher score)*	digital tip ulcers	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 ischemia, rather than trauma or exogenous causes.	2
	fingertip pitting scars		3
Telangiectasia	-	Visible macular dilated superficial blood vessels, which collapse upon pressure and fill slowly when pressure is released. Telangiectasia in a scleroderma-like pattern are round and well demarcated and found on hands, lips, inside of the mouth, ad/or are large mat-like telangiectasia. Distinguishable from rapidly filling spider angiomas with central arteriole and from dilated superficial vessels.	2

Table 3 | The ACR/EULAR 2013 classification criteria for systemic sclerosis *(continued)*

Item	subitem	Definition	weight/ score
abnormal nailfold capillaries	-	Enlarged capillaries and/or capillary loss with or without pericapillary haemorrhages at the nailfold. May also be seen on the cuticle.	2
pulmonary arterial hypertension and/or interstitial lung disease *(maximum score is 2)*	pulmonary arterial hypertension	pulmonary arterial hypertension diagnosed by right-sided heart catheterization according to standard definitions	
	interstitial lung disease	pulmonary fibrosis seen on high-resolution computed tomography or chest radiography, most pronounced in the basilar portions of the lungs, 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 hyperaemia in response to cold exposure or emotion; usually one phase is pallor.	3
SSc-related autoantibodies (maximum score is 3)	anti-centromere	positive according to lab standards	3
	anti-topoisomerase I		3
	anti-RNA polymerase III		3

1. These criteria are applicable to any patient considered for inclusion of an SSc study. The criteria are not applicable to patients with skin thickening sparing the fingers or to patients who have a scleroderma-like disorder that better explains their manifestations (e.g. nephrogenic sclerosis fibrosis, generalized morphea, eosinophilic fasciitis, scleredema diabeticorum, scleromyxedema, erythromelalgia, porphyria, lichen sclerosis, graft-versus-host disease, diabetic cheiropathy).
2. The total score is determined by adding the maximum weight (score) in each category. Patients with a total score of ≥9 are classified as having definite SSc.

Treatment of Systemic Sclerosis and the need for risk-stratification

Currently, treatment modalities in SSc are largely symptomatic and organ based. For example, nifedipine is used for Raynaud's phenomenon and iloprost is used for digital ulcers, although the available amount of evidence for all these treatment options are limited (53). As the disease is thought to be immune mediated, various trials with immunosuppressive and immune-regulatory agents have been and are being performed. Till date, no healing agents or therapeutic strategies that resolve organ damage have been identified. Below, current treatment recommendations for the major organ complications as defined by the EULAR are discussed (54).

For skin involvement two trials have shown that in early dcSSc, methotrexate might be beneficial (55, 56). Van den Hoogen et al. showed in a randomized control trial that in 17 patients receiving methotrexate (MTX), improvement of skin score (-0.7 (95%CI -3.4 to 2.1) over 24 weeks exceeded that of 12 patients in the placebo group (+1.2, 95%CI -1.2 to 3.5). The trial of Pope et al. showed no statistically significant difference in skin scores between patients treated with either methotrexate or placebo. A re-analysis of the results of this last trial by Pope et al., using Bayesian statistics showed that there is a 94% chance of a better skin score with MTX compared to placebo, with an estimated effect of -5.3 mRSS (95% credible interval -11.8 to 1.3). Although the discussion remains whether this is a clinical relevant difference (57) and whether this effect may be overestimated as the natural history of skin disease in SSc in most cases also involves improvement over time and groups were not entirely comparable (58). Nevertheless, currently MTX is the recommended treatment for isolated skin disease MTX.

For SSc-ILD, both cyclophosphamide IV and mycophenolate are the most common treatments. Two high-quality randomized controlled trials and their subanalyses have been performed, which have set the basis for cyclophosphamide treatment (31, 59). In the first Scleroderma Lung Study (SLS I) a placebo-corrected improvement in forced vital capacity (FVC) of 2.5% (95%CI 0.3 to 4.8) and total lung capacity (TLC) of 4.1% (95%CI 0.5 to 7.7) were found after treatment with oral cyclophosphamide. No significant effect on diffusion capacity of the lung for carbon monoxide (DLCO) could be demonstrated. As the modest changes raised questions about the clinical significance, a subanalysis evaluating high-resolution computed tomography (HRCT) was conducted (60). This study found significant treatment related changes in fibrosis scores on HRCT, that correlated with patient reported dyspnoea complaints. Extension of the SLS I study showed that after cessation of cyclophosphamide, the improvement in FVC continued, to finally reach a maximum 6 months after stopping. The beneficial effect disappeared 1 year after therapy was completed (32). Another subanalysis showed that skin disease and HRCT score were independent predictors of the response on cyclophosphamide (61). As the response might not be clinically relevant in all patients, risk-stratification is needed and only patients

likely to deteriorate towards severe disease should be considered for treatment. The fact that cyclophosphamide also comes with potential risks such as bone marrow suppression, teratogenicity, gonadal failure and haemorrhagic cystitis emphasize the need for stratification once more (62). The SLS I study itself however showed such risk-stratification isn't easy. The trial aimed at inclusion of patients likely to deteriorate in lung function during the trial period, however over a 1-year period also in the placebo group only a small change of -2.6±0.9 % predicted FVC was observed. Similar results were found in the trial described by Hoyles et al. in which the effect of intravenous cyclophosphamide was assessed (59).

Alternative to treatment with cyclophosphamide, mycophenolate mofetil may be used. The Scleroderma Lung Study II (SLS II) has shown that effects of mycophenolate mofetil are not inferior to treatment with oral cyclophosphamide (33).

Recently, additional treatment options have become available for SSc-ILD: Nintedanib, a tyrosine-kinase inhibitor, has shown to be able to slow deterioration rate of FVC. In a trial of 576 patients FVC decreased 52.4 ml per year for nintedanib group vs 93.3 ml per year with placebo; 95% confidence interval 2.9 to 79.0; P=0.04) (63). Nintedanib is considered mainly for patients with predominantly fibrotic lesions, rather than ground glass opacities (GGO) on their HRCT and in patients with longer standing disease (in which GGO is more likely to resemble subresolution of fibrotic changes instead of alveolar filling by inflammation), as the thought is that this agent is mainly calling a hold to the fibrotic process. This however still needs to be confirmed.

For patients with early-dcSSc (within 18 months of disease onset) with elevated acute phase-reactants and evidence of active disease, shown by presence of tendon-friction rubs and an increasing skin score, tocilizumab is a treatment option that might halt the disease process in the lungs (64, 65). In 210 patients randomized to either tocilizumab or placebo, tocilizumab treated patients had a stable FVC over 48 weeks of follow-up, while placebo treated patient showed a median of 5 points decline in FVC. However, in terms of treatment failures, there was no significant difference in patients having a >10% decrease in FVC during follow-up between the tocilizumab (13% vs 24%; HR 0.55 [95% CI 0.3-1.1]).

As PAH is a fatal complication, that occurs in about 10% of SSc patients and has a 5-year survival of 50% (66), early detection of PAH in SSc is important and for that purpose an algorithm – the DETECT score - was developed (67). Research that confirms the benefit of early detection and treatment or evaluating preventive treatments in SSc-PAH remains to be performed. Although PAH is a feared complication of SSc, trials in PAH often are not limited to SSc-PAH. Nevertheless, randomized controlled trials of endothelin receptor antagonists, PDE-5 inhibitors and riociguat, include also subgroup analyses of SSc-PAH patients. For this subgroup

these drugs show improvement of exercise capacity and prolonged time to clinical worsening (68-70). Therefore, treatment of SSc-PAH is similar to those of patients with idiopathic PAH and patients with other forms of CTD-PAH (71).

In severe cases of SSc, with a quick progressive disease course, the ultimate treatment of choice is autologous hematopoietic stem cell transplantation (HSCT). Two randomized controlled trials show clear beneficial effects of HSCT compared to treatment with IV cyclophosphamide with prolonged survival and less disease related complications. The ASTIS trial is a European multicenter trial conducted between March 2001 and October 2013, in which 156 patients with early diffuse SSc were randomized to either treatment with HSCT (n=79) or cyclophosphamide (n=77) (29). Van Laar et al. showed that HSCT was associated with increased event free-survival. Skin scores, FVC and total lung capacity improved significantly in HSCT treated patient. These findings were confirmed in the SCOT trial (28). Event-free survival here was 74% (total n=36) in HSCT treated patients versus 47% (total n=39) in cyclophosphamide treated patients. However, treatment with HSCT should not be performed at all costs: treatment related mortality is up to 10%. Because of this risk, patients having mild disease or patients in a relatively poorer condition (older patients and patients with severe cardiac or pulmonary involvement) are not eligible for this treatment.

The Leiden Multidisciplinary Systemic Sclerosis Care Pathway – “Combined Care In Systemic Sclerosis”

In 2014 the Dutch Society for Rheumatology (Nederlandse Vereniging voor Reumatologie; NVR) published a directive for the monitoring for Systemic Sclerosis, in the form of a care pathway (72). Prior to development of this care pathway, in 2009 in Leiden a multidisciplinary care pathway for SSc patients was started (73). Standardized and regular screening for organ involvement has shown to contribute to prolonged survival in SSc and justifies existence of care pathways in SSc (74).

‘The Leiden Multidisciplinary Systemic Sclerosis Care Pathway’ comprises an annual visit to the rheumatologist, pulmonologist and cardiologist. Additionally, extensive medical screening takes place and patients are seen by a physical therapist, specialized nurse, and, if requested, by social worker and/or occupational worker. Patients suspect for SSc, patients diagnosed with SSc in need of tertiary care because of disease severity and ‘shared care’ SSc patients from peripheral hospitals are seen in the Leiden Care Pathway. For every patient, the first care pathway is scheduled on two consecutive days, in which all appointments are between 8:00 and 16:00. During yearly follow-up, the content of the care pathway is more tailored and for some patients, the necessary screening can be performed on a single day. From initiation

in 2009 to the time being, the capacity of the care pathway has increased from 2 patients to 9 patients per week, with now over 1000 individual patients who visited the care pathway at least once.

As data of these prospectively followed patients have been entered in a research database, a unique cohort of patients has originated from ‘The Leiden Comprehensive Care Pathway’. With the initiation of a new database system, the research part of the “Leiden Multidisciplinary Care Pathway” has been named “Combined Care In Systemic Sclerosis” (CCISS). Data of this CCISS cohort form the basis of the work described in the current thesis.

Outline of the thesis

As disease specific antibodies are associated with distinct clinical phenotypes, several authors have suggested that monitoring SSc patients should be guided by antibody subtype (39, 41, 75). This assumption of antibodies as biomarker, suggests that SSc-specific auto-antibodies may function as the polar star for a captain at sea, in help of the physician determining the course for monitoring and treatment of the disease. In the current thesis we explore this hypothesis, with specific attention for anti-topoisomerase I antibodies.

In medicine, biomarkers facilitate early diagnosis, profile patients at risk for poor outcomes and may predict response to therapy. In **part I** of this thesis, we report the findings of a small clinical trial - the RITuximab In Systemic Sclerosis (RITIS) trial (**Chapter 2**). The trial could not confirm or reject potential efficacy of rituximab in SSc patients. The main learning point of the study was that currently in SSc, small clinical trials are difficult to interpret, as patient selection is highly complicated by the unpredictable disease course. It thereby demonstrates the high need for biomarkers in SSc, in order to select homogeneous and suitable patient groups for the outcomes of interest.

In **part II** the potential of autoantibodies to fulfil the biomarker need in SSc is evaluated. We show that autoantibody status only partially contributes to risk stratification in patients with SSc: not all ATA-positive patients have an infaust prognosis (**Chapter 3**) and although cancer risk is elevated in SSc, auto-antibodies alone cannot identify which patients to screen extensively for concurrent cancer (**Chapter 4**).

In **part III**, we focus on ATA+ SSc. We here show that the classic ATA auto-antibody association with severe progressive disease may be overrated. In **Chapter 5**, we show that as a result of improved identification of SSc patients using the ACR/EULAR 2013 classification criteria, also mild cases of ATA+ SSc are identified. Moreover,

a large deal of the classic associations made come from confounding by sex, as we show in **Chapter 6**. Nevertheless, we do show that immunologic characteristics of the auto-antibody response in SSc can be useful in clinical practice and may improve our understanding of pathophysiology in the future: Chapter 7 teaches us that when we specifically look at ATA-IgM auto-antibodies, this positivity associates with disease progression. This indicates that evaluating specific auto-antibodies responses in more detail perhaps can provide more guidance in disease management.

Finally, **part V** provides a summary and discussion of the results described in this thesis in **Chapter 8.**

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