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).

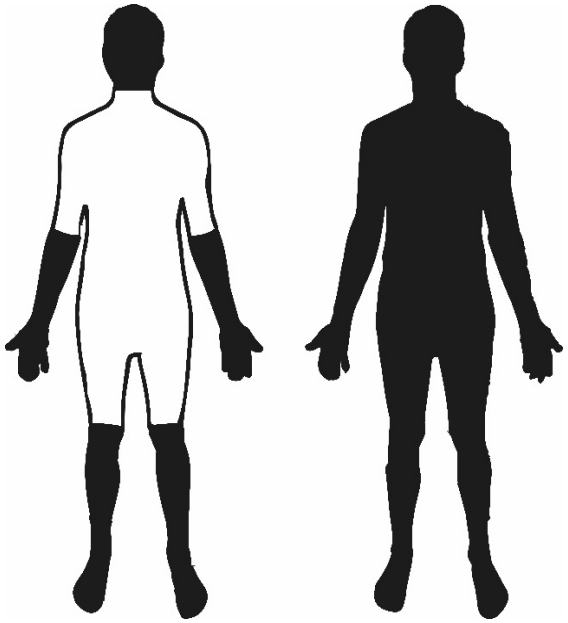

Figure 1. Limited (left) and diffuse (right) cutaneous Systemic Sclerosis

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).

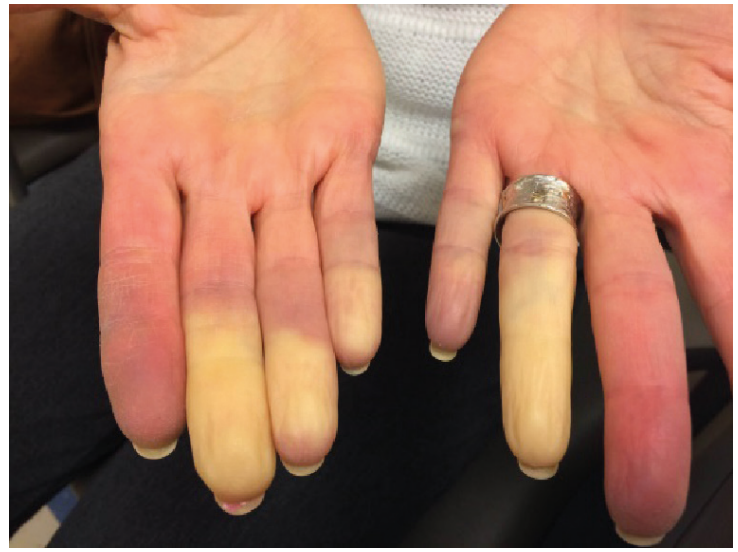

Figure 2. Raynaud's phenomenon –episodes of vasoconstriction of area's in the fingers, as a reaction to cold and/or emotion. Parts involved turn white and may turn blue and with return of blood flow red discoloration with burning sensation can occur.

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.

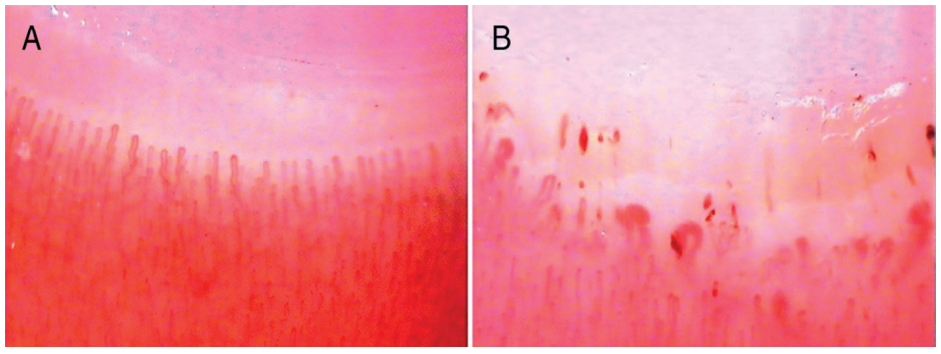

Figure 3. Nailfold microscopy images – left: a normally constructed nailbed, right: the nailbed of an SSc patient with less capillaries, enlarged capillaries and bleedings.

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.**

REFERENCES

1. Barnes J, Mayes MD. Epidemiology of systemic sclerosis: incidence, prevalence, survival, risk factors, malignancy, and environmental triggers. Curr Opin Rheumatol. 2012;24(2):165-70.
2. Andreasson 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. 2014;73(10):1788-92.
3. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Jr., et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol. 1988;15(2):202-5.
4. Elhai M, Meune C, Avouac J, Kahan A, Allanore Y. Trends in mortality in patients with systemic sclerosis over 40 years: a systematic review and meta-analysis of cohort studies. Rheumatology. 2011;51(6):1017-26.
5. Rodnan GP, Benedek TG. An historical account of the study of progressive systemic sclerosis (diffuse scleroderma). Ann Intern Med. 1962;57:305-19.
6. Lewin G, Heller J. Die sclerodermie. Berlin, A Hirschwald. 1895:169-71.
7. Osler LSW. The principles and practice of medicine. South Med J. 1930;23(11):1044.
8. Allanore Y, Simms R, Distler O, Trojanowska M, Pope J, Denton CP, et al. Systemic sclerosis. Nature reviews Disease primers. 2015;1:15002.
9. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. N Engl J Med. 2009;360(19):1989-2003.
10. Koenig M, Joyal F, Fritzler MJ, Roussin A, Abrahamowicz M, Boire G, et al. Autoantibodies and microvascular damage are independent predictive factors for the progression of Raynaud's phenomenon to systemic sclerosis: a twenty-year prospective study of 586 patients, with validation of proposed criteria for early systemic sclerosis. Arthritis Rheum. 2008;58(12):3902-12.
11. Maricq HR, Carwile LeRoy E. Patterns of finger capillary abnormalities in connective tissue disease by "wide-field" microscopy. Arthritis & rheumatology. 1973;16(5):619-28.
12. Fleischmajer R, Perlish JS. Capillary alterations in scleroderma. J Am Acad Dermatol. 1980;2(2):161-70.
13. Rodnan GP, Myerowitz RL, Justh GO. Morphologic changes in the digital arteries of patients with progressive systemic sclerosis (scleroderma) and Raynaud phenomenon. Medicine (Baltimore). 1980;59(6):393-408.
14. Kuwana M, Okazaki Y, Yasuoka H, Kawakami Y, Ikeda Y. Defective vasculogenesis in systemic sclerosis. The Lancet. 2004;364(9434):603-10.
15. Distler JH, Beyer C, Schett G, Luscher TF, Gay S, Distler O. Endothelial progenitor cells: novel players in the pathogenesis of rheumatic diseases. Arthritis Rheum. 2009;60(11):3168-79.
16. Avouac J, Juin F, Wipff J, Couraud PO, Chiocchia G, Kahan A, et al. Circulating endothelial progenitor cells in systemic sclerosis: association with disease severity. Ann Rheum Dis. 2008;67(10):1455-60.
17. Del Papa N, Pignataro F. The Role of Endothelial Progenitors in the Repair of Vascular Damage in Systemic Sclerosis. Front Immunol. 2018;9:1383.

18. Del Papa N, Quirici N, Soligo D, Scavullo C, Cortiana M, Borsotti C, et al. Bone marrow endothelial progenitors are defective in systemic sclerosis. Arthritis Rheum. 2006;54(8):2605-15.
19. Greenberg SD, O'Neal RM, Jenkins DE. The pathologic findings in diffuse interstitial fibrosis of the lungs. South Med J. 1974;67(5):571-9.
20. de Morree JJ. Dynamiek van het menselijk bindweefsel: functie, beschadiging en herstel: Bohn Stafleu Van Loghum; 2008.
21. Hinz B, Phan SH, Thannickal VJ, Prunotto M, Desmouliere A, Varga J, et al. Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. Am J Pathol. 2012;180(4):1340-55.
22. Manetti M, Guiducci S, Matucci-Cerinic M. The origin of the myofibroblast in fibroproliferative vasculopathy: does the endothelial cell steer the pathophysiology of systemic sclerosis? Arthritis Rheum. 2011;63(8):2164-7.
23. Kissin EY, Merkel PA, Lafyatis R. Myofibroblasts and hyalinized collagen as markers of skin disease in systemic sclerosis. Arthritis Rheum. 2006;54(11):3655-60.
24. Lafyatis R. Transforming growth factor beta-at the centre of systemic sclerosis. Nature Reviews Rheumatology. 2014;10(12):706-19.
25. Varga J, Abraham D. Systemic sclerosis: a prototypic multisystem fibrotic disorder. J Clin Invest. 2007;117(3):557-67.
26. Beyer C, Dees C, Distler JH. Morphogen pathways as molecular targets for the treatment of fibrosis in systemic sclerosis. Arch Dermatol Res. 2013;305(1):1-8.
27. Sakkas LI, Xu B, Artlett CM, Lu S, Jimenez SA, Platsoucas CD. Oligoclonal T cell expansion in the skin of patients with systemic sclerosis. J Immunol. 2002;168(7):3649-59.
28. Sullivan KM, Goldmuntz EA, Keyes-Elstein L, McSweeney PA, Pinckney A, Welch B, et al. Myeloablative Autologous Stem-Cell Transplantation for Severe Scleroderma. N Engl J Med. 2018;378(1):35-47.
29. van Laar JM, Farge D, Sont JK, Naraghi K, Marjanovic Z, Larghero J, et al. Autologous hematopoietic stem cell transplantation vs intravenous pulse cyclophosphamide in diffuse cutaneous systemic sclerosis: a randomized clinical trial. JAMA. 2014;311(24):2490-8.
30. Arkwright PD, Motala C, Subramanian H, Spergel J, Schneider LC, Wollenberg A. Management of difficult-to-treat atopic dermatitis. J Allergy Clin Immunol Pract. 2013;1(2):142-51.
31. Tashkin DP, Elashoff R, Clements PJ, Goldin J, Roth MD, Furst DE, et al. Cyclophosphamide versus placebo in scleroderma lung disease. N Engl J Med. 2006;354(25):2655-66.
32. Tashkin DP, Elashoff R, Clements PJ, Roth MD, Furst DE, Silver RM, et al. Effects of 1-year treatment with cyclophosphamide on outcomes at 2 years in scleroderma lung disease. Am J Respir Crit Care Med. 2007;176(10):1026-34.
33. Tashkin DP, Roth MD, Clements PJ, Furst DE, Khanna D, Kleerup EC, et al. Mycophenolate mofetil versus oral cyclophosphamide in scleroderma-related interstitial lung disease (SLS II): a randomised controlled, double-blind, parallel group trial. The Lancet Respiratory Medicine. 2016.
34. White B, Moore WC, Wigley FM, Xiao HQ, Wise RA. Cyclophosphamide is associated with pulmonary function and survival benefit in patients with scleroderma and alveolitis. Ann Intern Med. 2000;132(12):947-54.

35. Jordan S, Distler JH, Maurer B, Huscher D, van Laar JM, Allanore Y, et al. Effects and safety of rituximab in systemic sclerosis: an analysis from the European Scleroderma Trial and Research (EUSTAR) group. Ann Rheum Dis. 2015;74(6):1188-94.
36. Moroi Y, Peebles C, Fritzler MJ, Steigerwald J, Tan EM. Autoantibody to centromere (kinetochore) in scleroderma sera. Proc Natl Acad Sci U S A. 1980;77(3):1627-31.
37. Tan EM, Rodnan GP, Garcia I, Moroi Y, Fritzler MJ, Peebles C. Diversity of antinuclear antibodies in progressive systemic sclerosis. Anti-centromere antibody and its relationship to CREST syndrome. Arthritis Rheum. 1980;23(6):617-25.
38. Douvas AS, Achten M, Tan EM. Identification of a nuclear protein (Scl-70) as a unique target of human antinuclear antibodies in scleroderma. J Biol Chem. 1979;254(20):10514-22.
39. Steen VD. Autoantibodies in systemic sclerosis. Semin Arthritis Rheum. 2005;35(1):35-42.
40. Steen VD, Powell DL, Medsger TA, Jr. Clinical correlations and prognosis based on serum autoantibodies in patients with systemic sclerosis. Arthritis Rheum. 1988;31(2):196-203.
41. Nihtyanova SI, Denton CP. Autoantibodies as predictive tools in systemic sclerosis. Nat Rev Rheumatol. 2010;6(2):112-6.
42. Association AR. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Arthritis Rheum. 1980;23(5):581-90.
43. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/ European League against Rheumatism collaborative initiative. Arthritis Rheum. 2013;65(11):2737-47.
44. Finckh A, Liang MH, van Herckenrode CM, de Pablo P. Long-term impact of early treatment on radiographic progression in rheumatoid arthritis: A meta-analysis. Arthritis Rheum. 2006;55(6):864-72.
45. Robinson PC, Brown MA. The window of opportunity: a relevant concept for axial spondyloarthritis. Arthritis Res Ther. 2014;16(3):109.
46. Matucci-Cerinic M, Bellando-Randone S, Lepri G, Bruni C, Guiducci S. Very early versus early disease: the evolving definition of the 'many faces' of systemic sclerosis. Ann Rheum Dis. 2013;72(3):319-21.
47. van Steenbergen HW, van Nies JA, Huizinga TW, Bloem JL, Reijnierse M, van der Helm-van Mil AH. Characterising arthralgia in the preclinical phase of rheumatoid arthritis using MRI. Ann Rheum Dis. 2015;74(6):1225-32.
48. van den Berg R, de Hooge M, van Gaalen F, Reijnierse M, Huizinga T, van der Heijde D. Percentage of patients with spondyloarthritis in patients referred because of chronic back pain and performance of classification criteria: experience from the Spondyloarthritis Caught Early (SPACE) cohort. Rheumatology (Oxford). 2013;52(8):1492-9.
49. Minier T, Guiducci S, Bellando-Randone S, Bruni C, Lepri G, Czirják L, et al. Preliminary analysis of the Very Early Diagnosis of Systemic Sclerosis (VEDOSS) EUSTAR multicentre study: evidence for puffy fingers as a pivotal sign for suspicion of systemic sclerosis. Ann Rheum Dis. 2014;73(12):2087-93.
50. Ingegnoli F, Boracchi P, Gualtierotti R, Smith V, Cutolo M, Foeldvari I, et al. A comparison between nailfold capillaroscopy patterns in adulthood in juvenile and adult-onset systemic sclerosis: A EUSTAR exploratory study. Microvasc Res. 2015;102:19-24.

51. Weiner ES, Hildebrandt S, Senecal JL, Daniels L, Noell S, Joyal F, et al. Prognostic significance of anticentromere antibodies and anti-topoisomerase I antibodies in Raynaud's disease. A prospective study. Arthritis Rheum. 1991;34(1):68-77.
52. Spencer-Green G. Outcomes in primary Raynaud phenomenon: a meta-analysis of the frequency, rates, and predictors of transition to secondary diseases. Arch Intern Med. 1998;158(6):595-600.
53. Nagaraja V, Denton CP, Khanna D. Old medications and new targeted therapies in systemic sclerosis. Rheumatology (Oxford). 2015;54(11):1944-53.
54. Kowal-Bielecka O, Fransen J, Avouac J, Becker M, Kulak A, Allanore Y, et al. Update of EULAR recommendations for the treatment of systemic sclerosis. Ann Rheum Dis. 2017;76(8):1327-39.
55. Johnson SR, Feldman BM, Pope JE, Tomlinson GA. Shifting our thinking about uncommon disease trials: the case of methotrexate in scleroderma. J Rheumatol. 2009;36(2):323-9.
56. Pope JE, Bellamy N, Seibold JR, Baron M, Ellman M, Carette S, et al. A randomized, controlled trial of methotrexate versus placebo in early diffuse scleroderma. Arthritis Rheum. 2001;44(6):1351-8.
57. Khanna D, Furst DE, Hays RD, Park GS, Wong WK, Seibold JR, et al. Minimally important difference in diffuse systemic sclerosis: results from the D-penicillamine study. Ann Rheum Dis. 2006;65(10):1325-9.
58. Herrick AL, Pan X, Peytrignet S, Lunt M, Hesselstrand R, Mouthon L, et al. Treatment outcome in early diffuse cutaneous systemic sclerosis: the European Scleroderma Observational Study (ESOS). Ann Rheum Dis. 2017;76(7):1207-18.
59. Hoyles RK, Ellis RW, Wellsbury J, Lees B, Newlands P, Goh NS, et al. A multicenter, prospective, randomized, double-blind, placebo-controlled trial of corticosteroids and intravenous cyclophosphamide followed by oral azathioprine for the treatment of pulmonary fibrosis in scleroderma. Arthritis Rheum. 2006;54(12):3962-70.
60. Goldin J, Elashoff R, Kim HJ, Yan X, Lynch D, Strollo D, et al. Treatment of scleroderma-interstitial lung disease with cyclophosphamide is associated with less progressive fibrosis on serial thoracic high-resolution CT scan than placebo: findings from the scleroderma lung study. Chest. 2009;136(5):1333-40.
61. Roth MD, Tseng CH, Clements PJ, Furst DE, Tashkin DP, Goldin JG, et al. Predicting treatment outcomes and responder subsets in scleroderma-related interstitial lung disease. Arthritis Rheum. 2011;63(9):2797-808.
62. Lynch JP, 3rd, McCune WJ. Immunosuppressive and cytotoxic pharmacotherapy for pulmonary disorders. Am J Respir Crit Care Med. 1997;155(2):395-420.
63. Distler O, Highland KB, Gahlemann M, Azuma A, Fischer A, Mayes MD, et al. Nintedanib for Systemic Sclerosis-Associated Interstitial Lung Disease. N Engl J Med. 2019;380(26):2518-28.
64. Khanna D, Denton CP, Jahreis A, van Laar JM, Frech TM, Anderson ME, et al. Safety and efficacy of subcutaneous tocilizumab in adults with systemic sclerosis (faSScinate): a phase 2, randomised, controlled trial. Lancet (London, England). 2016;387(10038):2630-40.
65. Khanna D, Lin CJF, Furst DE, Goldin J, Kim G, Kuwana M, et al. Tocilizumab in systemic sclerosis: a randomised, double-blind, placebo-controlled, phase 3 trial. The Lancet Respiratory medicine. 2020;8(10):963-74.

66. Hachulla E, Carpentier P, Gressin V, Diot E, Allanore Y, Sibilia J, et al. Risk factors for death and the 3-year survival of patients with systemic sclerosis: the French ItinerAIR-Sclerodermie study. Rheumatology (Oxford). 2009;48(3):304-8.
67. Coghlan JG, Denton CP, Grünig E, Bonderman D, Distler O, Khanna D, et al. Evidence-based detection of pulmonary arterial hypertension in systemic sclerosis: the DETECT study. Ann Rheum Dis. 2014;73(7):1340-9.
68. Pulido T, Adzerikho I, Channick RN, Delcroix M, Galie N, Ghofrani HA, et al. Macitentan and morbidity and mortality in pulmonary arterial hypertension. N Engl J Med. 2013;369(9):809-18.
69. Ghofrani HA, Galie N, Grimminger F, Grunig E, Humbert M, Jing ZC, et al. Riociguat for the treatment of pulmonary arterial hypertension. N Engl J Med. 2013;369(4):330-40.
70. Humbert M, Coghlan JG, Ghofrani HA, Grimminger F, He JG, Riemekasten G, et al. Riociguat for the treatment of pulmonary arterial hypertension associated with connective tissue disease: results from PATENT-1 and PATENT-2. Ann Rheum Dis. 2017;76(2):422-6.
71. Ghofrani HA, Distler O, Gerhardt F, Gorenflo M, Grunig E, Haefeli WE, et al. Treatment of pulmonary arterial hypertension (PAH): updated Recommendations of the Cologne Consensus Conference 2011. Int J Cardiol. 2011;154 Suppl 1:S20-33.
72. Jeurissen M, Nijmegen SM, Knaapen-Hans H, Nijmegen R, Meijs J, Leiden U, et al. Zorgpad Systemische Sclerose (SSc).
73. Meijs J, Schouffoer AA, Ajmone Marsan N, Kroft LJ, Stijnen T, Ninaber MK, et al. Therapeutic and diagnostic outcomes of a standardised, comprehensive care pathway for patients with systemic sclerosis. RMD open. 2016;2(1):e000159.
74. Nihtyanova SI, Tang EC, Coghlan JG, Wells AU, Black CM, Denton CP. Improved survival in systemic sclerosis is associated with better ascertainment of internal organ disease: a retrospective cohort study. QJM. 2010;103(2):109-15.
75. Kuwana M. Circulating Anti-Nuclear Antibodies in Systemic Sclerosis: Utility in Diagnosis and Disease Subsetting. Journal of Nippon Medical School = Nippon Ika Daigaku zasshi. 2017;84(2):56-63.

PART I

The need for biomarker research

2

Rituximab in early Systemic Sclerosis

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RMD Open. 2017 Jul 28; 3

Abstract

Objectives
1) Hypothesis testing of the potency of rituximab in preventing fibrotic complications and 2) assessing acceptability and feasibility of rituximab in early Systemic Sclerosis.

Methods
A small, 24-month, randomized, double-blind, placebo-controlled, single-centre trial in Systemic Sclerosis patients diagnosed <2 years, was conducted. Patients received rituximab or placebo infusions at t= 0, t = 15 days and t = 6 months. Patients were clinically evaluated every three months, with lung function tests and HRCT every other visit. Skin biopsies were taken at baseline and month 3. Immunophenotyping of peripheral blood mononuclear cells was performed at every visit, except at month 9 and 18. Adverse events, course of skin and pulmonary involvement and B cell populations in skin and peripheral blood were evaluated.

Results
In total 16 patients (rituximab n=8, placebo n=8) were included. Twelve patients had diffuse cutaneous systemic sclerosis. Eighty-eight adverse events (rituximab n=53, placebo n=35, p=0.22) and 11 serious adverse events (rituximab n=7, placebo n=4, p=0.36) occurred. No unexpected rituximab related events were observed. Mean skin score over time did not differ between the groups. Over time, FVC and extent of lung involvement slightly improved with rituximab, but this difference was insignificant. In peripheral blood B cells depletion was demonstrated.

Conclusions
No unexpected safety issues were observed with rituximab in early Systemic Sclerosis. Although this small trial could not confirm or reject potential efficacy of rituximab in these patients, future placebo controlled trials are warranted, specifically in the subgroup of patients with pulmonary involvement.

Trial registration: www.clinicaltrialsregister.eu, EudraCT Number: 2008-07180-16

Introduction

Systemic sclerosis (SSc) is an autoimmune disease that is characterized by the triad of microvascular damage, dysregulation of innate and adaptive immunity, and generalized fibrosis in multiple organs(1) .

The pathogenesis of SSc is poorly understood and treatment is organ and symptom based. Current therapy targeting the dysregulated immune system, supported by clinical trial data, includes methotrexate for early skin involvement (2, 3), cyclophosphamide followed by mycophenolate mofetil or azathioprine for lung involvement (4-8) and autologous hematopoietic stem cell transplantation for severe, diffuse cutaneous Systemic Sclerosis (dcSSc) (9).

Experimental data suggested a key role for B cells in the pathogenesis of SSc(10). B cells seem to overexpress the stimulatory receptor CD19 and IL-10-producing regulatory B cells are decreased (11).

Previous observational open-label studies of anti-CD20 therapy (rituximab) and a nested-case control study in SSc showed potential efficacy for skin disease and stabilization of internal organ disease in dcSSc (12-21). Since natural disease course is variable and difficult to predict, these results are difficult to value and need to be replicated in randomized controlled trials (22).

We hypothesized that the window-of opportunity for rituximab (RTX) in SSc patients lies early in the disease course, when fibrotic complications are yet to develop. This hypothesis is based on observations in a study in mice in which B cell depletion with anti-CD20 was effective in prevention of skin fibrosis in new-born tight-skin mice while no benefit was observed in tight skin mice with established disease (23). Additionally, BAFF levels in these mice were elevated at 4 weeks after birth, while normalized at week 12 when skin fibrosis was established (24).

Based on these observations, we aimed to test the hypothesis that RTX can prevent development of severe fibrotic complications in early Systemic Sclerosis. Additionally, safety and feasibility of rituximab in early SSc is described, together with the influence of rituximab on immune cell subsets in peripheral blood and in skin tissue in SSc patients.

Methods

Trial design
The rituximab in early systemic sclerosis (RITIS) trial was designed as a 24-month, parallel, double-blind, placebo-controlled randomized trial. Randomization was performed in a 1:1 ratio by the Pharmacy of the Leiden University Medical Center (LUMC), Leiden, The Netherlands. Ethical approval was obtained from the Medical Ethical Committee (METC) of the LUMC and patients gave written informed consent. The study was monitored by a Data and Safety Monitoring Board until completion.

Patients
Between June 2010 and February 2014, patients with an established diagnosis of SSc according to the American Rheumatology Association (ARA) criteria (25) within the last 24 months before enrolment and aged between 18 and 70 years were included. Previous immunosuppressive therapy was allowed and continued throughout the trial. Patients with a history of deep tissue infection within 1 year prior to baseline, patients with chronic or recurrent infections and patients with a history of cancer were excluded.

Procedures
Patients received IV 1000mg rituximab (Mabthera©/Anti CD 20 mAb) or placebo (0.9% NaCl) on day 1 and day 15 as induction treatment. Consolidation treatment consisted of a single IV treatment with 1000mg rituximab or placebo (0.9% NaCl) at 6 months. Each infusion of rituximab was given together with methylprednisolone 100mg IV, oral paracetamol 1000mg and clemastine 2mg IV. Placebo treated patients received 1.6mL 0.9% NaCl together with oral paracetamol 1000mg and clemastine 2mg IV. Concomitant medications or other treatments deemed necessary for patients' supportive care and safety were allowed at the discretion of the treating physician. Patients, physicians and the observers performing the skin score were blinded for treatment allocation.

Data collection
Patients were seen every 3 months during the first year and every 6 months thereafter, for physical examination including the modified Rodnan skin score (mRSS), and assessing toxicity (National Cancer Institute, Common toxicity parameters (CTC))(26), urine analyses and laboratory testing(at t=0, 3,6, 12, and 24 months also including samples for immunophenotyping of peripheral blood) for a total follow-up of 24 months. Skin scores were assessed by an experienced research nurse (AV) and a research physician who was trained by AV (JM). In two-thirds of cases the skin score was assessed by AV and in one third by JM. Patients filled out the following

questionnaires at every visit: Short Form 36 (SF-36) (27, 28), EuroIQol-5D (EQ-5D) (29, 30) and Health Assessment Questionnaire Disability Index (HAQ-DI)(31, 32). Lung function tests including Forced Vital Capacity (FVC) and Diffusing capacity of the Lungs for carbon monoxide (DLCO), High-resolution computed tomography (HRCT) of the thorax and echocardiography were performed every 6 months. HRCT's were assessed using Goh criteria evaluating the extent of lung involvement at five levels: 1) origin of great vessels; 2) main carina; 3 pulmonary venous confluence; 4) halfway between the third and fifth section; 5) immediately above the right-diaphragm.(33) Scoring was performed consensus based, by 2 observers (AS and LK).

For histologic and immunohistochemical analysis of the skin, 4 mm skin biopsies were obtained from the dorsal side of the forearm, within 1 cm of each other, at baseline and at 3 months.

Immunohistochemistry of skin tissue was performed on 4µm thick sections on polylysine-coated slides. After routine deparaffinization and rehydration, antigens were retrieved in a tissue microwave for 12 min at 98°C with a Target Retrieval Solution Tris/EDTA pH 9. Quenching of endogenous peroxidase activity was performed with 1% hydrogen peroxide in methanol for 10 minutes. Biopsies were incubated with: Haematoxylin and eosin staining (4085.9005 and 4085.9002; Klinipath; Duiven, Netherlands) (general histopathology assessment and mononuclear infiltration), PBS/1%BSA for 1hour with CD3 (1.41 µg/ml; M7254; DAKO) (T-cells), CD68 (0.12 µg/ml; M0814; DAKO) (macrophages), CD79a (1.875 µg/ml; M7050; DAKO) (B-cells including plasma cells). Human tonsil specimens were used as a positive control for antibodies.

Stained sections were coded and scored by three observers, who were unaware of clinical data and treatment regimen (AD, KQ, MB) with respect to the following points: histologic signs of scleroderma skin (such as presence and entrapment of adnexa), mononuclear infiltration (semi-quantitative scale), T cell infiltration (semi-quantitative scale), B cell infiltration (semi-quantitative scale) and macrophage infiltration (semi-quantitative scale). Semi-quantitative scoring for lymphocyte and macrophages was based on the scoring scale for lymphocytes proposed by Roumm et al. with '0' being a few scattered cells, '1' a maximum number of cells per collection of at least 10, '2' a maximum number of cells per collection between 10 and 50 and '3' a maximum number of cells per collection of at least 50(34). Median scores were used for analysis.

Peripheral blood mononuclear cells (PBMCs) were isolated from 50 mL of peripheral blood by Ficoll-Paque gradient centrifugation, incubated for 20 minutes at 4°C and subsequently stained with CD3 APC (clone SK7), CD4 FITC (clone RPA-T4), CD8 PE (clone RPA-T8), CD14 FITC (clone MSE2), CD16 PE (clone B73.1), CD19 PerCPcy5.5 and APC (clone Sj25C1), CD20 FITC (clone L27), CD27 PE (clone L128), CD38 PerCPcy5.5 (clone HIT2), CD56 PE (clone MY31), Polyclonal IgA FITC (DAKO), IgD FITC (clone IA6-

2), IgE Alexa Fluor 488 (gift from University of Antwerp), IgG FITC (clone G18-145), IgM APC and FITC (clone G20-127, all (except IgA and IgE) BD Biosciences. For isotype controls, IgG1 APC and PerCPcy5.5 (clone MOPC-21), IgG1 FITC and PE (clone X40), IgG2a FITC (clone X39), IgG2b FITC (clone 27-35), Rabbit immunoglobulin fraction (DAKO), Polyclonal Swine anti-Rabbit Immunoglobulins FITC (DAKO), all (Rabbit immunoglobulins) BD Biosciences, were used. In addition, B cells and plasmablasts were stained with CD20, IgA, IgD, IgE, IgG, IgM and appropriate isotype controls.

ELISPOT technique was used to detect functional antibody-secreting cells, with the use of goat anti–human IgG, IgA and IgM (Sanbio BV, Uden, The Netherlands) for coating (10 µg/ml in coating buffer, 100 µl/well) of ELISPOT plates (Millipore, The Netherlands). Plates were incubated overnight at 4°C, washed twice with PBS and blocked with 200 µl/well culture medium (IMDM + 10% FCS + 200 mM L-glutamine + 100 µg/ml penicillin/streptavidin) for 2 hours at 37°C in a 5% CO2 atmosphere. PBMCs were titrated on the ELISPOT plates in duplicate wells, and the plates were next incubated at 37°C in a 5% CO2 atmosphere overnight. The following day cells were discarded and washed from the plates with PBS/0.05% Tween 20 and tap water. Spots were visualised by detection with alkaline phosphatase-conjugated goat anti-human IgG, IgM or IgA (Biosource, USA) followed by substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich, St Louis, MO, USA) at 100 µl/well. Enzyme-linked immunosorbent spots (Elispots) were analysed using a stereomicroscope (Bioreader 5000; BIO-SYS GmbH, Karben, Germany.

Study end points

In the design of the trial the following parameters were defined as major clinical end points: treatment related mortality, toxicity and clinical efficacy of rituximab. Efficacy was defined as progression-free survival, with progression defined as any or a combination of the following changes relative to baseline at two consecutive evaluations: death, ≥ 10% drop in predicted FVC(33), ≥ 15% drop in predicted DLCO(33), ≥15% drop in left ventricular ejection fraction (LVEF), body weight(35), ≥ 30% drop in creatinine clearance(36), ≥ 30% increase in mRSS(37, 38), ≥ 0.5 point increase in HAQ-DI(38). The secondary end points defined were changes in mRSS (minimally important difference 3.2-5.3)(39), FVC, DLCO, HAQ-DI (minimally important difference 0.10-0.14)(39), left ventricular ejection fraction, creatinine clearance, SF 36, EuroQol 5 D, presence of interstitial lung disease as reported by HRCT thorax and skin biopsy scores.

However, unfortunately, the trial had major recruitment problems. In a time span of nearly 4 years, 17 patients had been included in the trial. Based on this low inclusion rate the METC advised to prematurely end inclusion and evaluate study outcome 1 year after inclusion of the last patient. As one patient showed early drop-out, n=16 patients (n=8 rituximab, and n= 8 placebo) had data available for analysis. All data collected by June 30th, 2015 were included in the analysis. Based on the small

sample size, we chose to focus on presentation of changes in mRSS, FVC, DLCO and extent of ILD as represented by Goh scores. Adverse events and serious adverse events and changes in HAQ-DI, LVEF, creatinine clearance, SF 36, EuroQol 5 D were assessed for both treatment groups. Immunologically, the influence of rituximab on mononuclear cell subsets in PBMC's and skin tissue was evaluated and described as planned.

Statistical analysis

As all patients participating in the trial also participated in the care program of the LUMC (40), including annual and comprehensive diagnostic evaluation with informed consent for use of data, missing data were imputed from clinical files when possible with a maximum time frame of 6 months between data collection and planned data collection according to the trial schedule. This way skin scores were available up till 24 months for all patients, pulmonary function tests for n=13/16 of patients at t=12 months and n=7/16 at t=24 months. HRCT images were available for scoring in n=15/16 at t=12 months and n=7/16 at t=24 months. Peripheral blood samples for PBMC assessment were available in n=15/18 at t=12 months and n=11/18 at t= 24 months.

Primary analyses included mean change over time over time in mRSS, percentage of predicted DLCO, percentage of predicted FVC, extent of ILD as represented by Goh scores and HAQ-DI, for both treatment groups. Additionally, mortality, treatment toxicity and efficacy according to pre-specified criteria were evaluated for both groups.

Ninety-five percent confidence intervals were computed where appropriate, with p-values less than 0.05 (2-sided) considered statistically significant. Binary variables were analysed by Fisher exact test.

To assess the influence on clinical efficacy analyses of patients included under protocol violation, analyses were repeated excluding these patients. Inter-observer agreement of skin biopsy scoring was evaluated using Fleiss kappa (41). Statistical analyses were performed using IBM SPSS Statistics 23 and GraphPad Prism 6.

Results

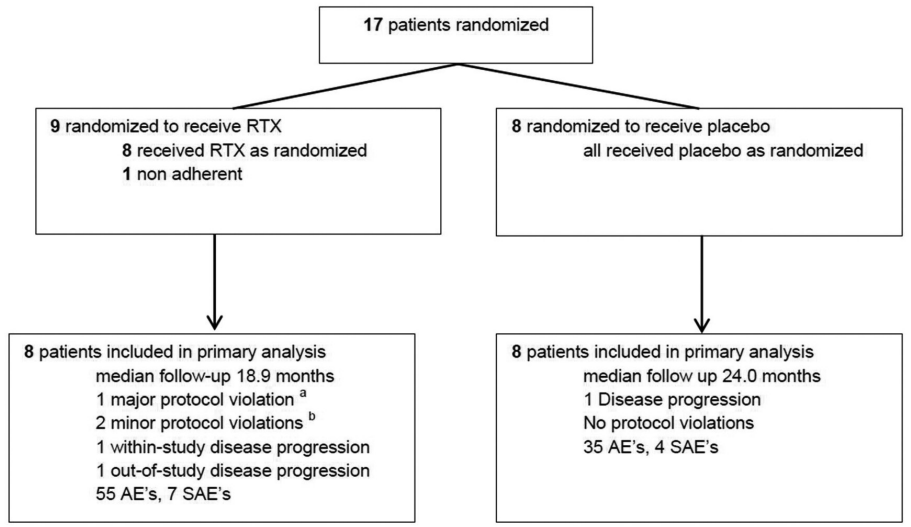
Patients and treatment

From April 2010 to February 2014, 17 patients were included, of which 9 patients were randomized to rituximab and 8 were randomized to placebo (Fig. 1). All patients included fulfilled ARA criteria as well as ACR/EULAR 2013 SSc criteria (42). Two patients were included with a time since diagnosis of > 24 months: one rituximab patient (time since diagnosis 3.5 years, time since non-Raynaud 3.5 years and time since Raynaud 5.3 years) and one placebo patient (time since diagnosis 4.2 years,

time since non-Raynaud 4.2 years and time since Raynaud 4.9 years). One patient (placebo group) died due to disease progression, after drop-out at 6 months because of active disease. One patient did not start the allocated treatment based on active, severe disease with rapid progression of skin score and myocarditis/pericarditis, for which the treating physician judged the chance for placebo as possibly life threatening. Baseline characteristics of this patient did not differ from other patients included.

Baseline characteristics of the 16 patients included for analysis (rituximab n=8, placebo n=8) were similar between the 2 groups, though there was a minor difference in disease duration, with slightly longer disease duration in the placebo group (Table 1). The median follow-up of patients was 19.1 months (IQR 17.6 – 24.4). According to Goh criteria, mean extent of lung involvement at baseline was 9.5%±11.0 for RTX and 6.9% ±10.8 for placebo (p=0.65).

Figure 1 Flow of RITIS (Rituximab in Scleroderma) trial



RTX; rituximab, AE; adverse event, SAE; serious adverse event

[a] One patient refused to have a treatment at 6 months

[b] Two patients incidentally received a dose of verum methylprednisolone instead of placebo methylprednisolone together with the RTX/placebo infusion

Previous immunosuppressive therapy included prednisone (RTX n=2, placebo n=1), methotrexate (RTX n=3, placebo n=0) and azathioprine (RTX n=2, placebo n=0). At start of the trial use of immunosuppressive medication included prednisone (RTX n=2, placebo n=0), methotrexate (RTX n=5, placebo n=3), plaquenil (RTX n=1, placebo n=1), mycophenolate mofetil (RTX=1, placebo n=1). During the trial background immunosuppressive treatment was changed in 2 patients in the rituximab group: methotrexate was stopped at the 18 month visit in both cases (n=1: pregnancy; n=1: pancytopenia).

Table 1 Baseline characteristics of study patients

Characteristic	**RTX group** (n = 8)	**placebo group** (n = 8)	**p-value**
Demographic			
age, mean (yr.)	44.5±5.6	36.6±4.3	0.21 [a]
female sex (% of patients, n)	87.5 (7)	87.5 (7)	1 [b]
caucasians (% of patients, n)	75 (6)	62.5 (5)	0.58 [b]
Disease specific			
dcSSc (% of patients, n)	87.5 (7)	62.5 (5)	0.57 [b]
duration of scleroderma (yr.)			
since diagnosis, (median, range)	0.9 (0.7-3.5)	1.3 (0.2-4.2)	0.44 [a]
since onset first Raynaud symptom (median, range)	2.3 (0.7-5.3)	4.3 (0.7-16.1)	0.13 [a]
since onset first non-Raynaud symptom (median, range)	1.2 (0.6-3.5)	2.4 (0.7-4.2)	0.25 [a]
Skin and musculoskeletal			
modified Rodnan Skin Score (mean±SE)	16.4±4.4	14.0±3.8	0.88 [a]
Heart and Lungs			
LVEF (mean±SE)	61.1±4.2	62.0±4.6	0.96 [a]
FVC (% of predicted)	97.9±6.6	92.0±6.1	0.67 [a]
DLCO (% of predicted)	67.1±4.2	72.3±6.0	0.34 [a]
Total extent of lung disease on HRCT (mean %)	9.5±11.0	6.9±10.8	0.65 [a]
Extent ground glass (mean %)	8.3±9.4	5.4±8.0	0.44 [a]
Extent reticular pattern (mean %)	4.0±8.7	3.9±7.1	1 [a]
Function and Quality of Life			
HAQ-DI (mean±SE)	1.39±0.27	1.31±0.32	0.65 [a]

Table 1 Baseline characteristics of study patients *(continued)*

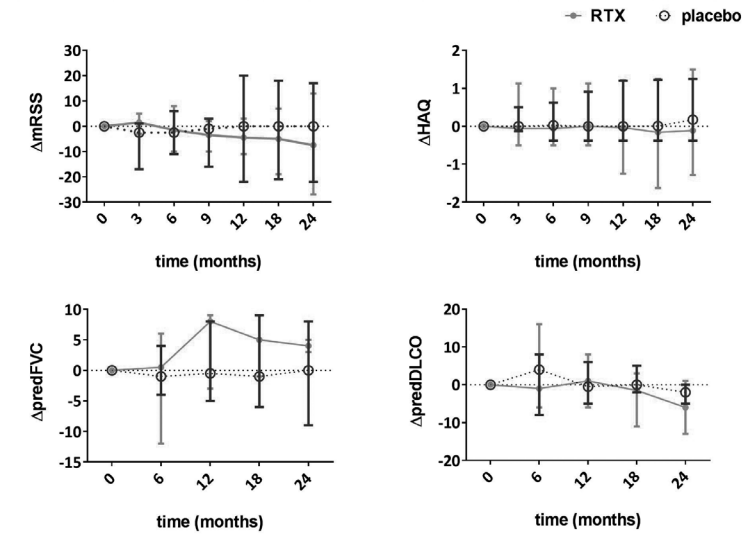
Characteristic	**RTX group** (n = 8)	**placebo group** (n = 8)	**p-value**
Therapy			
Previous immunosuppressive therapy* (% of patients)	50.0	12.5	0.28 [b]
months of use (median, range)	1.5 (0.0-36.0)	0.0 (0.0-9.0)	0.20 [a]
Immunosuppressive therapy** (% of patients)	87.5	62.5	0.57 [b]
months of use (median, range)	8.1(0.0-42.6)	3.2 (0.0-26.3)	0.33 [a]
Laboratory findings			
ANA-positive (% of patients)	100	87.5	1.00[b]
anti-topoisomerase I (% of patients)	12.5	50.0	0.28 [b]
anti-RNA polymerase III (% of patients)	25.0	0.0	0.47 [b]

dcSSc; diffuse cutaneous systemic sclerosis, RTX; rituximab [a] : Mann-Whitney [b] : Fisher's exact
*Previous immunosuppressive therapy included high-dose (> 15mg/day) prednisone (RTX n=2, placebo n=1), methotrexate (RTX n=3, placebo n=0) and azathioprine (RTX n=2, placebo n=0).
**Current immunosuppressive treatment included high-dose (> 15mg/day) prednisone (RTX n=2, placebo n=0), methotrexate (RTX n=5, placebo n=3), plaquenil (RTX n=1, placebo n=1), mycophenolate mofetil (RTX=1, placebo n=1).
*** Extent of lung disease in HRCT was scored according to Goh criteria (33) ; the extent was evaluated over five levels and averaged (1 origin of great vessels; 2 main carina; 3 pulmonary venous confluence; 4. halfway between the third and fifth section; 5.immediately above the right hemi-diaphragm)

Analysis of clinical disease parameters

Course of changes in mRSS, FVC, DLCO and HAQ are shown in Figure 2. There were no significant differences in change between baseline and 12-month follow-up of mRSS (placebo -1.8 vs. RTX -3.6, p=0.95), FVC (placebo+0.3 vs. RTX +4.7, p=0.43), DLCO (placebo -0.3 vs. +0.7, p=0.91) and HAQ (placebo +0.18 vs. RTX 0.0, p=0.94). Also, at 24-month follow-up, there were no significant differences in change from baseline in mRSS (placebo -1.9 vs. RTX -5.3, p=0.95), FVC (placebo -1.4 vs. RTX +4, p=0.65), DLCO (placebo -2.2, RTX -6.0, p=0.77) and HAQ (placebo 0.2313 vs. RTX -0.0675, p=0.94) results. Numerically, n=4/8 rituximab vs. n=2/8 in placebo improved >5 points in mRSS, there were no improvers in either FVC or DLCO (minimal important difference 10%) and n=1/8 in rituximab vs. n=0/8 in placebo improved in HAQ (minimal important difference 0.5 points) after one year.

Figure 2 Change in modified Rodnan Skin Score, Forced Vital Capacity, Diffusing Capacity of the Lung and Health Assesment Questionnaire during 24 month follow up in patients with systemic sclerosis treated with rituximab or placebo



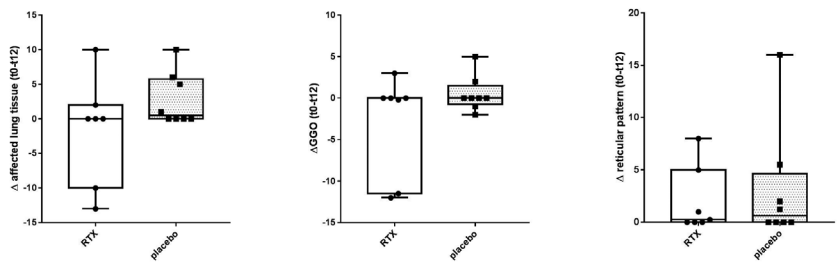
RTX; rituximab – mRSS; modified Rodnan Skin Score – predFVC; forced vital capacity, percentage of predicted – predDLCO; diffusing capacity of the lung; percentage of predicted - HAQ; Health Assessment Questionnaire - Dots indicate medians, error bars indicate ranges

Analysis of HRCT data according to Goh criteria showed a mean change in percentage of affected lung tissue between baseline and 12 months of -1.6% for rituximab and + 2.8% for placebo (p=0.28). Beneficial effects were explained by a decrease in ground glass opacities with rituximab treatment in two rituximab treated patients (Fig 3+4). Numerically, n=2/7 rituximab patients and n=0/8 placebo patients showed improvement on HRCT (-10% or more change in mean extent of lung involvement), n=4/7 in rituximab vs. n=7/8 in placebo had stable lung involvement on HRCT (between -10% and +10% change in mean extent of lung involvement) and n=1/7 in rituximab vs. n=1/8 in placebo had worsening lung involvement on HRCT (+10% or more change in extent of lung involvement).

Analysis of change in AUC showed no significant differences in the mRSS, FVC and HAQ-DI between the groups (Supp. Table S2). Within the first year, mean change from baseline to 12 months follow-up in mRSS was comparable between groups, with -1.4 for the rituximab and -2.7 for the control group (difference 1.3; 95%CI -3.4 to 6.2; p=0.55). For FVC and HAQ-DI differences in AUC between baseline and one year were also insignificant. For FVC there was a slight improvement with rituximab and a slight detoriation with placebo (mean change AUC baseline to 12 months follow-up 0.6 for RTX and -0.4 for placebo, p=0.59). Also during the second year, no significant differences were observed in AUC for mRSS, FVC and HAQ-DI (Supp. Table S2).

Efficacy analyses for the individual disease parameters were repeated excluding the two patients with disease duration > 24 months since diagnosis. These analyses did not show different results.

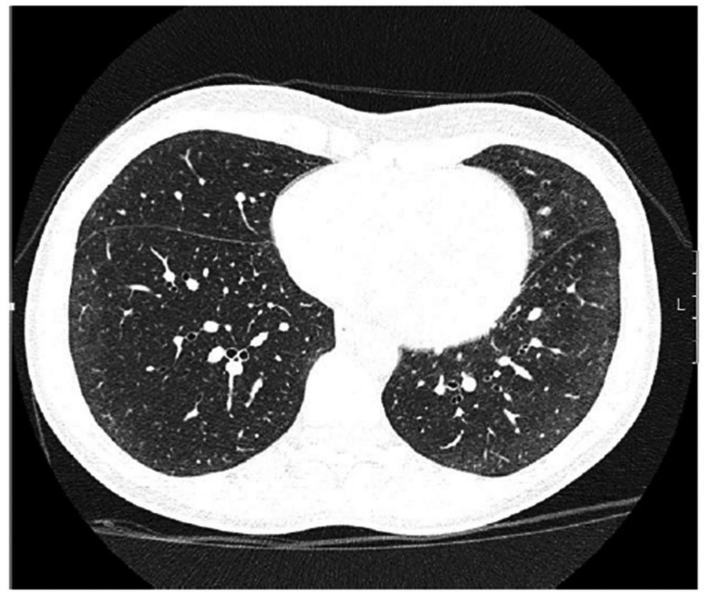
Figure 3 Change in mean extent of lung tissue and lung tissue with ground glass or reticular pattern involvement from baseline to 12 months follow-up in patients with Systemic Sclerosis



RTX; rituximab – t0;baseline – t12;12 months follow-up
vertical axis represent differences in Goh scores between baseline and 12 months for (from left to right): 1) mean extent of affected lung tissue, 2) GGO; mean extend of ground glass opacities, 3) mean extend of affected lung tissue with reticular pattern.

At 12 months a non-significant trend in favour of rituximab was observed (mean change in lung involvement: -1.6% RTX group vs. +2.8% placebo group [p=0.28]). The beneficial effect in the rituximab group was explained by a decrease of ground glass opacities.

Figure 4 Improvement of lung involvement as evaluated by HRCT of a patient treated with rituximab



HRCT High Resolution Computed Tomography; inspiratory scan, halfway between pulmonary venous confluence and right hemi-diaphragm

Upper HRCT: baseline HRCT before rituximab use, lower HRCT: 12 months after initial gift of rituximab

Recorded parameters reflecting vascular complications did not differ between the treatment arms: no patients in the trial had impaired kidney function and eGFR rates within study arms were comparable (mean eGFR at T=0: placebo 118.5 ml/min/1.73m2, RTX 106.8 ml/min/1.73m2) and were stable throughout the trial. Also LVEF (as measured by echocardiography) remained stable throughout the trial in all participants (mean LVEF at T=0: placebo 62.0%, RTX 60.2%, at T=24: placebo 60.2%, RTX 65.5%). Digital ulcers occurred both in the placebo (n=3) and the rituximab group (n=3).

In the analyses SF-36 scores and EQ-5D scores no differences were seen (data not shown).

Two patients in the placebo group showed disease progression during follow-up according to pre-specified criteria, including the patient that died after drop-out. In the rituximab group one patient showed disease progression. Apart from the patient that died, study disease progression was based on a ≥30% increased mRSS relative to baseline at the 12-month (placebo, n=1) and 18-month visits (RTX, n=1) in both cases. Including the patient that died after drop-out, there was no difference in progression free survival between groups (Log Rank (Mantel-Cox) p=0.674). Also after excluding the two patients with disease duration > 24 months there was no significant difference in progression free survival between the groups.

Safety and toxicity

No patients died during the study. One patient (placebo group) died due to disease progression, after drop-out at 6 months because of active disease. This patient eventually died at 23 months due to scleroderma renal crisis after autologous hematopoietic stem cell transplantation.

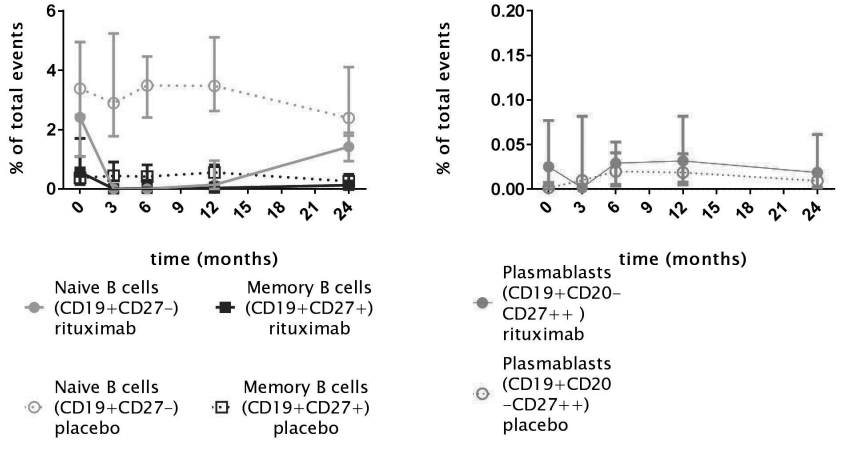
A total of 88 adverse events (AE's) occurred during the study: 52 in the rituximab group (6 grade 3 AE's, 2 grade 4 AE's) and 36 in the control group (7 grade 3 AE's, 0 grade 4 AE's)(p=0.22)(Supp. Table S1). There were 7 serious adverse events (SAE's) in the rituximab group and 4 in the placebo group (p=0.36). Serious adverse events in the rituximab group were a breast carcinoma (18 months after 1st gift of RTX), abnormal cervical histology leading to hysterectomy (6 months after first gift of RTX; medical history of this patient mentioned abnormal cervical histology also before inclusion in the trial), an anaemia due to severe menstruation (7 months after 1st gift of RTX), a pancytopenia (12 months after 1st gift of RTX) and 3 events related to digital ulcers (n=2 at 1 month after 1st gift of RTX, the other at 7 months). Serious adverse events in the placebo group included severe weight loss which required treatment by percutaneous endoscopic gastrostomy placement (17 months after first gift of RTX) and 3 events related to digital ulcers (1, 14 and 18 months after initial RTX). There were more grade 1 AE's in the rituximab group due to mild infusion reactions (system organ class type: immune system). A clear causal relation between adverse events and treatment with rituximab could not be established, except for mild infusion reactions.

Immunophenotyping of peripheral blood mononuclear cells

At baseline there were no differences seen in proportions of macrophages (CD14+), NK cells (CD56+), T helper cells (CD3+/CD4+), cytotoxic T cells (CD3+/CD8+) or B cells (CD19+) between placebo and rituximab group (Data not shown). Three months after initial anti-CD20 treatment significant depletion B cells was seen and simultaneously a decline in T cells was observed. Counter wise the proportion of macrophages increased.

When observing the different subsets of B cells during the study, as shown in Figure 5, naïve and memory B cells were depleted 3 months after the first gift of rituximab. In 5 of 8 patients with rituximab a reduction of CD19+CD20-CD27++ plasmablasts was seen. Reduction of plasmablasts was significant within the rituximab group when compared to baseline, but insignificant when compared to the placebo at the same time point. When assessing depletion of immunoglobulin expressing (IgG, IgA, IgM and IgD) naïve (CD19+CD27-) and memory (CD19+CD27+) subsets, as a positive control, all subsets were depleted (Data not shown). At time of consolidation treatment (month 6), repopulation of naïve B cells, memory B cells and plasmablasts was present. However, throughout the complete follow-up period repopulation of naïve B cells and memory B cells was incomplete in the rituximab group (Figure 5).

Figure 5 Naïve B cells (CD19+CD27 -), memory B cells (CD19+CD27+) and plasmablasts (PB CD27++) levels in patients with systemic sclerosis



Indicated points resemble medians; Rituximab: T0, n= 8; T3 n=8; T6 n=7; T12 n=6; T24 n=4; Placebo: T0, n=8; T3, n=8; T6 n=8; T12 n=7; T24 n=7
B cell depletion after rituximab treatment is seen in all B cell subsets
Total events are defined as the number of detected cells by the flow cytometer.

Skin biopsies
Skin biopsies were performed in 15 patients (RTX n=7, placebo n=8) at baseline and in 14 patients (RTX n=7, placebo n=7) at 3 months (Supp. Table S3). For 3 patients in the placebo group, and for 1 patient in the rituximab group, skin was clinically unaffected at the site of biopsy Inter-observer agreement of histologic skin score evaluated by Fleiss kappa was κ =0.49 for T cells, κ=0.32 for B cells and κ=0.63 for macrophages.

There were no significant differences found in immune cell presence in skin neither between groups, nor within groups over time. At baseline there was a trend towards more mononuclear infiltration in the placebo group, based on the presence of more T cells. Over time, presence of T cells in the rituximab group increased at 3 months compared to baseline. Presence of other immune cells was stable over time (Supp. Table S3.).

B cells were rarely present in skin tissue, only 1 patient in the placebo group that showed a collection of >10 cells, but less than 50 cells at baseline. Scattered B cells (range 2-7 per biopsy) were seen in 5 out of 15 biopsies at baseline (RTX n=3, placebo n=2). Over time, there were no changes in the presence of B cells in skin of rituximab treated patients, 3 months after initial gift, with B cells present in 4 out of 7 biopsies. This was identical to the number of placebo patients with B cell presence in skin at three months (4 out of 7).

Discussion

This small randomized, placebo controlled trial cannot reject nor confirm the hypothesis of RTX preventing fibrotic complications. No major safety issues were observed with rituximab in the subset of early SSc patients. Immunologically, rituximab achieved its presumed biological effect: a depletion of circulating B cells up to minimal counts, but with persistence of antigen secreting cells and incomplete depletion of the CD27++ plasmablast compartment. No change in the small number of cells from the B cell lineage present in skin tissue was observed with rituximab treatment. Over time, small, non-significant differences in FVC, extent of pulmonary involvement and HAQ-DI in favour of the rituximab group were found. Further research must confirm the credibility of these findings. A larger scale RCT in patients with proven pulmonary involvement therefore seems plausible and feasible.

Unfortunately, the trial had recruitment problems resulting in premature termination of inclusion. Moreover, patients in the control group experienced an unexpected favourable disease course, which complicates firm conclusions about efficacy of rituximab in preventing fibrotic complications. This study aimed to include patients with early dcSSc. Indeed, our placebo group included patients of which the majority

had dcSSc at baseline (63%), and 4 of 8 patients were either ATA or RNApIII positive. Both these antibodies are associated with more severe disease course (43, 44). Despite these characteristics reflecting high risk, early dcSSc, 75% of patients in the placebo group had favourable outcome after 2 years.

There is a small insignificant difference in disease duration between the rituximab and the placebo group, with the placebo group having a longer disease duration. It has been shown that with longer disease duration, chances to improve spontaneously slightly increase(45) which might partially explain the beneficial disease course in placebo. However, excluding the two patients with the longest disease duration did not change our results.

Several case reports, open-label studies and a nested case-control study thus far reported a potential beneficial effect of rituximab on pulmonary function, skin fibrosis and functional impairment in SSc (12-21, 46-49). Our observations are in line with the study from Lafyatis (15), who treated 15 patients with early SSc with rituximab and did not find a clear beneficial effect on skin fibrosis and pulmonary function at 6 and 12 months of follow-up. Various explanations can account for the difference between previous open-label studies and our findings. As these studies did not include a placebo group, part of the observed efficacy might reflect natural disease course. In addition, most open-label studies included patients with longer disease duration (13, 14, 50) and thus possibly selected an immunologically different subgroup of SSc patients. In comparison to the open label studies of Smith and Lafyatis (15, 17), who both also included patients with early disease, mean skin scores were lower in our population, which complicates the possibility of demonstrating clear clinical efficacy on skin involvement. When analysing only the patients with dcSSc at baseline, with rituximab n=3/7 showed a decrease in mRSS >5, versus n=2/5 within the placebo group. On the other hand, it is known that patients with more skin fibrosis at baseline are more likely to regress even without therapy over the next year (51).

Immunophenotyping of peripheral blood showed almost complete B cell depletion, which is in line with previous studies (15, 17). It is known that during treatment with rituximab, plasmablasts and plasma cells can persist (52). Besides confirming this with ELISPOT, thereby showing persistence of IgA, IgG and IgM antigen secreting cells after treatment with rituximab, this is also demonstrated by the incomplete depletion of the CD27++ plasmablast compartment (Supp Figure S1). Other studies found CD20-positive B cells in skin biopsies in approximately half of patients at baseline and depletion in most of these cases (12, 13, 17). To overcome possible interference of rituximab treatment with detection of CD20-positive B cells in skin we chose to use CD79a staining on skin, which also stains plasmablasts and plasma cells that lack CD20, while in previous studies CD20 staining was used. Based on morphology, the persisting B-cells in our samples could reflect unaffected long-lived

B cell populations. The exact nature and the relevance of these persistent B cells for development and persistence of skin fibrosis remains to be determined, and might be relevant in determining subsets of SSc patients with high likelihood of responding to rituximab.

Remarkably, 2 out of 8 rituximab treated patients showed evident improvement of the extent of ground glass opacities in HRCT at 1 year follow-up versus none of the placebo treated patients. Radiologic improvement of CTD-associated and RA-ILD after treatment with rituximab has also been described by other authors(53, 54). Out of interest, and to possibly guide future research in the field we compared different B cell subsets in baseline PBMC's between rituximab patients with pulmonary improvement and those without. Rituximab treated patients with pulmonary improvement both had higher counts of naïve B cells (CD19+,CD27-) counts of naïve B cells (CD19+, CD27-) (mean CD19+CD27+ of n=2 non-improvers under RTX 1.8% of total events vs. n=6 improvers under RTX 5.6% of total events, p=0.003), while other subsets were comparable. We speculate that this subgroup of patients, possibly reflecting those with very early and active inflammatory pulmonary involvement might be the subset of patients most likely to benefit from B cell depleting therapy. However, these observations obviously await replication.

In conclusion, we performed a double blind placebo controlled trial in patients with early SSc and show in-depth analysis of B cells in peripheral blood and skin tissue. Although given the small sample size and the unexpected favourable disease course in the placebo group no firm conclusions on clinical efficacy of rituximab in early SSc can be drawn, our data show that a larger RCT in early SSc with proven pulmonary involvement might be worthwhile. In addition, inclusion of peripheral blood and skin tissue analyses is also warranted in future trials to determine the nature, role and relevance of persisting B-cells in skin, and persisting plasmablasts and plasma cells in peripheral blood. Analysis as presented herein might help to identify a subset of SSc patients most likely to benefit from B cell depleting therapies.

Supporting information

Supplementary data is available at the website of RMD Open or can be obtained by contacting the first author

References

1. Allanore Y, Simms R, Distler O, Trojanowska M, Pope J, Denton CP, et al. Systemic sclerosis. Nature Reviews Disease Primers. 2015:15002.
2. Johnson SR, Feldman BM, Pope JE, Tomlinson GA. Shifting our thinking about uncommon disease trials: the case of methotrexate in scleroderma. The Journal of rheumatology. 2009;36(2):323-9.
3. Pope JE, Bellamy N, Seibold JR, Baron M, Ellman M, Carette S, et al. A randomized, controlled trial of methotrexate versus placebo in early diffuse scleroderma. Arthritis Rheum. 2001;44(6):1351-8.
4. Tashkin DP, Elashoff R, Clements PJ, Goldin J, Roth MD, Furst DE, et al. Cyclophosphamide versus placebo in scleroderma lung disease. The New England journal of medicine. 2006;354(25):2655-66.
5. Hoyles RK, Ellis RW, Wellsbury J, Lees B, Newlands P, Goh NS, et al. A multicenter, prospective, randomized, double-blind, placebo-controlled trial of corticosteroids and intravenous cyclophosphamide followed by oral azathioprine for the treatment of pulmonary fibrosis in scleroderma. Arthritis Rheum. 2006;54(12):3962-70.
6. Derk CT, Grace E, Shenin M, Naik M, Schulz S, Xiong W. A prospective open-label study of mycophenolate mofetil for the treatment of diffuse systemic sclerosis. Rheumatology (Oxford, England). 2009;48(12):1595-9.
7. Gerbino AJ, Goss CH, Molitor JA. Effect of mycophenolate mofetil on pulmonary function in scleroderma-associated interstitial lung disease. Chest. 2008;133(2):455-60.
8. Nagaraja V, Denton CP, Khanna D. Old medications and new targeted therapies in systemic sclerosis. Rheumatology (Oxford, England). 2015;54(11):1944-53.
9. van Laar JM, Farge D, Sont JK, Naraghi K, Marjanovic Z, Larghero J, et al. Autologous hematopoietic stem cell transplantation vs intravenous pulse cyclophosphamide in diffuse cutaneous systemic sclerosis: a randomized clinical trial. Jama. 2014;311(24):2490-8.
10. Sakkas LI, Bogdanos DP. Systemic sclerosis: New evidence re-enforces the role of B cells. Autoimmun Rev. 2016;15(2):155-61.
11. Mavropoulos A, Simopoulou T, Varna A, Liaskos C, Katsiari CG, Bogdanos DP, et al. Breg Cells Are Numerically Decreased and Functionally Impaired in Patients With Systemic Sclerosis. Arthritis & rheumatology (Hoboken, NJ). 2016;68(2):494-504.
12. Bosello S, De Santis M, Lama G, Spano C, Angelucci C, Tolusso B, et al. B cell depletion in diffuse progressive systemic sclerosis: safety, skin score modification and IL-6 modulation in an up to thirty-six months follow-up open-label trial. Arthritis Res Ther. 2010;12(2):R54.
13. Daoussis D, Liossis SN, Tsamandas AC, Kalogeropoulou C, Kazantzi A, Sirinian C, et al. Experience with rituximab in scleroderma: results from a 1-year, proof-of-principle study. Rheumatology (Oxford, England). 2010;49(2):271-80.
14. Jordan S, Distler JH, Maurer B, Huscher D, van Laar JM, Allanore Y, et al. Effects and safety of rituximab in systemic sclerosis: an analysis from the European Scleroderma Trial and Research (EUSTAR) group. Annals of the rheumatic diseases. 2015;74(6):1188-94.
15. Lafyatis R, Kissin E, York M, Farina G, Viger K, Fritzler MJ, et al. B cell depletion with rituximab in patients with diffuse cutaneous systemic sclerosis. Arthritis Rheum. 2009;60(2):578-83.

16. Smith V, Piette Y, van Praet JT, Decuman S, Deschepper E, Elewaut D, et al. Two-year results of an open pilot study of a 2-treatment course with rituximab in patients with early systemic sclerosis with diffuse skin involvement. The Journal of rheumatology. 2013;40(1):52-7.
17. Smith V, Van Praet JT, Vandooren B, Van der Cruyssen B, Naeyaert JM, Decuman S, et al. Rituximab in diffuse cutaneous systemic sclerosis: an open-label clinical and histopathological study. Annals of the rheumatic diseases. 2010;69(1):193-7.
18. Moazedi-Fuerst FC, Kielhauser SM, Brickmann K, Hermann J, Lutfi A, Meilinger M, et al. Rituximab for systemic sclerosis: arrest of pulmonary disease progression in five cases. Results of a lower dosage and shorter interval regimen. Scandinavian journal of rheumatology. 2014;43(3):257-8.
19. Bosello SL, De Luca G, Rucco M, Berardi G, Falcione M, Danza FM, et al. Long-term efficacy of B cell depletion therapy on lung and skin involvement in diffuse systemic sclerosis. Semin Arthritis Rheum. 2015;44(4):428-36.
20. Daoussis D, Liossis SN, Tsamandas AC, Kalogeropoulou C, Paliogianni F, Sirinian C, et al. Effect of long-term treatment with rituximab on pulmonary function and skin fibrosis in patients with diffuse systemic sclerosis. Clin Exp Rheumatol. 2012;30(2 Suppl 71):S17-22.
21. Moazedi-Fuerst FC, Kielhauser SM, Bodo K, Graninger WB. Dosage of rituximab in systemic sclerosis: 2-year results of five cases. Clinical and experimental dermatology. 2015;40(2):211-2.
22. Mendoza FA, Keyes-Elstein LL, Jimenez SA. Systemic sclerosis disease modification clinical trials design: quo vadis? Arthritis Care Res (Hoboken). 2012;64(7):945-54.
23. Hasegawa M, Hamaguchi Y, Yanaba K, Bouaziz JD, Uchida J, Fujimoto M, et al. B-lymphocyte depletion reduces skin fibrosis and autoimmunity in the tight-skin mouse model for systemic sclerosis. The American journal of pathology. 2006;169(3):954-66.
24. Matsushita T, Fujimoto M, Hasegawa M, Matsushita Y, Komura K, Ogawa F, et al. BAFF antagonist attenuates the development of skin fibrosis in tight-skin mice. Journal of Investigative Dermatology. 2007;127(12):2772-80.
25. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Arthritis Rheum. 1980;23(5):581-90.
26. Institute' NC. Common Terminology Criteria for Adverse Events (CTCAE). USDEPARTMENT OF HEALTH AND HUMAN SERVICES.
27. Ware JJS, K.; Kosinki, M. Health survey manual and interpretation guide.1994.
28. Newnham EA, Harwood KE, Page AC. Evaluating the clinical significance of responses by psychiatric inpatients to the mental health subscales of the SF-36. Journal of Affective Disorders. 2007;98(1):91-7.
29. Dolan P. Modeling valuations for EuroQol health states. Medical care. 1997;35(11):1095-108.
30. Lamers L, Stalmeier P, McDonnell J, Krabbe P, Van Busschbach J. Kwaliteit van leven meten in economische evaluaties: het Nederlands EQ-5D-tarief. Nederlands tijdschrift voor geneeskunde. 2005;149(28):1574-8.
31. Fries JF, Spitz P, Kraines RG, Holman HR. Measurement of patient outcome in arthritis. Arthritis & Rheumatism. 1980;23(2):137-45.

32. Clements PJ, Wong WK, Hurwitz EL, Furst DE, Mayes M, White B, et al. The Disability Index of the Health Assessment Questionnaire is a predictor and correlate of outcome in the high-dose versus low-dose penicillamine in systemic sclerosis trial. Arthritis & Rheumatism. 2001;44(3):653-61.
33. Goh NS, Desai SR, Veeraraghavan S, Hansell DM, Copley SJ, Maher TM, et al. Interstitial lung disease in systemic sclerosis: a simple staging system. American journal of respiratory and critical care medicine. 2008;177(11):1248-54.
34. Roumm AD, Whiteside TL, Medsger TA, Jr., Rodnan GP. Lymphocytes in the skin of patients with progressive systemic sclerosis. Quantification, subtyping, and clinical correlations. Arthritis Rheum. 1984;27(6):645-53.
35. Baron M, Bernier P, Côté L, Delegge M, Falovitch G, Friedman G, et al. Screening and management for malnutrition and related gastro-intestinal disorders in systemic sclerosis: recommendations of a North American expert panel. Clin Exp Rheumatol. 2010;28(Suppl 58):S42-S6.
36. Penn H, Howie A, Kingdon E, Bunn C, Stratton R, Black C, et al. Scleroderma renal crisis: patient characteristics and long-term outcomes. Qjm. 2007;100(8):485-94.
37. Clements P, Lachenbruch P, Siebold J, White B, Weiner S, Martin R, et al. Inter and intraobserver variability of total skin thickness score (modified Rodnan TSS) in systemic sclerosis. The Journal of rheumatology. 1995;22(7):1281-5.
38. Gazi H, Pope JE, Clements P, Medsger TA, Martin RW, Merkel PA, et al. Outcome measurements in scleroderma: results from a delphi exercise. The Journal of rheumatology. 2007;34(3):501-9.
39. Khanna D, Furst DE, Hays RD, Park GS, Wong WK, Seibold JR, et al. Minimally important difference in diffuse systemic sclerosis: results from the D-penicillamine study. Annals of the rheumatic diseases. 2006;65(10):1325-9.
40. Meijs J, Schouffoer AA, Ajmone Marsan N, Kroft LJ, Stijnen T, Ninaber MK, et al. Therapeutic and diagnostic outcomes of a standardised, comprehensive care pathway for patients with systemic sclerosis. RMD open. 2016;2(1):e000159.
41. Fleiss JL. Measuring nominal scale agreement among many raters. Psychological bulletin. 1971;76(5):378.
42. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/ European League against Rheumatism collaborative initiative. Arthritis Rheum. 2013;65(11):2737-47.
43. Graf SW, Hakendorf P, Lester S, Patterson K, Walker JG, Smith MD, et al. South Australian Scleroderma Register: autoantibodies as predictive biomarkers of phenotype and outcome. Int J Rheum Dis. 2012;15(1):102-9.
44. Nihtyanova S, Tang E, Coghlan J, Wells A, Black C, Denton C. Improved survival in systemic sclerosis is associated with better ascertainment of internal organ disease: a retrospective cohort study. Qjm. 2010;103(2):109-15.
45. Steen VD, Medsger TA. Severe organ involvement in systemic sclerosis with diffuse scleroderma. Arthritis & Rheumatism. 2000;43(11):2437-44.
46. Daoussis D, Liossis SN, Tsamandas AC, Kalogeropoulou C, Kazantzi A, Korfiatis P, et al. Is there a role for B-cell depletion as therapy for scleroderma? A case report and review of the literature. Semin Arthritis Rheum. 2010;40(2):127-36.

47. Giuggioli D, Lumetti F, Colaci M, Fallahi P, Antonelli A, Ferri C. Rituximab in the treatment of patients with systemic sclerosis. Our experience and review of the literature. Autoimmun Rev. 2015;14(11):1072-8.
48. McGonagle D, Tan AL, Madden J, Rawstron AC, Rehman A, Emery P, et al. Successful treatment of resistant scleroderma-associated interstitial lung disease with rituximab. Rheumatology (Oxford, England). 2008;47(4):552-3.
49. Haroon M, McLaughlin P, Henry M, Harney S. Cyclophosphamide-refractory scleroderma-associated interstitial lung disease: remarkable clinical and radiological response to a single course of rituximab combined with high-dose corticosteroids. Therapeutic advances in respiratory disease. 2011;5(5):299-304.
50. Giuggioli D, Lumetti F, Colaci M, Fallahi P, Antonelli A, Ferri C. Rituximab in the treatment of patients with systemic sclerosis. Our experience and review of the literature. Autoimmun Rev. 2015;14(11):1072-8.
51. Dobrota R, Maurer B, Graf N, Jordan S, Mihai C, Kowal-Bielecka O, et al. Prediction of improvement in skin fibrosis in diffuse cutaneous systemic sclerosis: a EUSTAR analysis. Annals of the rheumatic diseases. 2016.
52. Mei HE, Frolich D, Giesecke C, Loddenkemper C, Reiter K, Schmidt S, et al. Steady-state generation of mucosal IgA+ plasmablasts is not abrogated by B-cell depletion therapy with rituximab. Blood. 2010;116(24):5181-90.
53. Sharp C, McCabe M, Dodds N, Edey A, Mayers L, Adamali H, et al. Rituximab in autoimmune connective tissue disease–associated interstitial lung disease. Rheumatology. 2016:kew195.
54. Md Yusof M, Kabia A, Darby M, Lettieri G, Beirne P, Vital E, et al. Effect of rituximab on the progression of rheumatoid arthritis-related interstitial lung disease: 10 years' experience at a single centre. Rheumatology (Oxford, England). 2017.

PART II

Auto-antibodies as biomarkers in Systemic Sclerosis

3

To what extent do auto-antibodies help to identify high-risk patients in Systemic Sclerosis?

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Abstract

Objective
To evaluate the additive value of auto-antibodies in identifying Systemic Sclerosis (SSc) patients with high complication risk.

Methods
Patients entering the Combined Care In SSc cohort, Leiden University Medical Center between April 2009 and May 2016 were included. Subgroups of patients were determined using hierarchical clustering, performed on Principal Component Analysis scores , 1) using baseline data of demographic and clinical variables only and 2) with additional use of antibody status. Disease-risk within subgroups was assessed by evaluating 5-year mortality rates. Clinical and auto-antibody characteristics of obtained subgroups were compared.

Results
In total 407 SSc patients were included of which 91% (n=371) fulfilled ACR/EULAR 2013 criteria for SSc. Prevalences of auto-antibodies were anti-centromere 37%, anti-topoisomerase (ATA) 24%, anti-RNA polymerase III 5%, anti-fibrillarin 4% and anti-Pm/Scl 5%. Clinical cluster analysis identified 4 subgroups, with two subgroups showing higher than average mortality (resp. 17% and 7% vs. total group mortality of 4%). ATA-positivity ranged from 10 to 21% in low-risk groups and from 30 to 49% among high-risk groups . Adding auto-antibody status to the cluster process resulted in 5 subgroups with 3 showing higher than average mortality. Still, 22% of ATA-positive patients were clustered into a low-risk subgroup, while the total number of patients stratified to a high-risk subgroup increased.

Conclusion
Auto-antibodies only partially contribute to risk-stratification and clinical subsetting in SSc. The current findings confirm that not all ATA-positive patients have worse prognosis and as such, additional biomarkers are needed to guide clinical follow-up in SSc.

Introduction

Systemic Sclerosis (SSc) is a disease that can affect almost any organ (1). Skin fibrosis is characteristic, but also interstitial lung disease (ILD), gastro-intestinal involvement and peripheral vasculopathy are common. Disease complications such as myositis, renal crisis, cardiac disease and pulmonary arterial hypertension (PAH) are less frequent, though require monitoring as they are associated with increased mortality (2). Five-year survival is approximately 89% for incident cases, with PAH and ILD being leading causes of death (3). Identification of patients with high disease-risk by identification of biomarkers, remains a topic of ongoing research(4). Currently, patients are monitored tight when disease is thought to be progressive based on: modified Rodnan Skin score (mRSS) ≥20, progressive skin scores, tendon friction rubs or anti-topoisomerase antibodies (ATA) (5).

Within the traditional subclassification based on skin involvement, non-cutaneous and limited cutaneous (lcSSc) are associated with better prognosis and PAH, while diffuse cutaneous (dcSSc) is associated with poorer prognosis, ILD and renal crisis (5, 6).

Different mutually exclusive disease-specific auto-antibodies are known, which can possibly guide disease monitoring (7). For anti-centromere antibody (ACA) monitoring with focus on PAH has been opted. Similarly, for ATA complete work-up for at least the first 4 years after diagnosis is advocated with pulmonary function tests (PFT) and high resolution computer tomography (HRCT) every 3-6 months, because of the association with severe ILD (7).

In contrast, the additive value of auto-antibodies in risk-prediction for the individual patient remains unclear. This is for example demonstrated by a recent study on PAH prediction, where the presence of ACA is suggested to predict PAH in an entire SSc population, but not in a model restricted to lcSSc patients, possibly because of the strong relation of lcSSc and ACA (8).

Moreover, in the clinical setting the physician can rely on a high number of clinical variables other than auto-antibody status, possibly of help in risk-stratification. Currently, auto-antibody status is of additional value for risk-stratification in prevalent disease and evaluated in several previous studies as such(5, 9-12). However, by evaluating the combination of clinical characteristics with auto-antibody status, the actual contribution of the auto-antibody to risk-stratification is partially blurred. Knowledge of the specific contribution of the auto-antibody can on the one hand improve clinical risk-stratification, and on the other hand shed light on the actual pathophysiological role of the auto-antibody itself. Therefore, we aimed to create subgroups based on comprehensive clinical information, including information on not only skin, but also musculoskeletal, cardiac, pulmonary and gastro-intestinal

complaints at cohort entry, as well as demographic data and assess disease-risk using available follow-up data. We took advantage of our well described, prospective SSc cohort with annual and complete clinical data available and subsequently performed cluster analysis with and without additional inclusion of auto-antibody status to evaluate additive value of auto-antibody status next to comprehensive clinical data.

Materials and methods

Patient selection
Data of 407 patients with a clinical diagnosis of SSc (91% (n=371) fulfilled ACR/EULAR 2013 criteria for SSc(13)) included in the Combined Care In Systemic Sclerosis cohort (CCISS cohort; Leiden Systemic Sclerosis Cohort) between April 1st 2009 and May 1st 2016 were used for analysis. Ethical approval for data collection was obtained from the Institutional Review Board of the LUMC. As described previously, all patients undergo annual extensive medical screening during a 2-day health care program (14).

Clinical variables
The following demographic and clinical variables were included in the cluster analyses: 1) demographic and disease-specific: sex, age, length, weight, time since first onset Raynaud phenomenon, time since onset first non-Raynaud phenomenon, diffuse SSc (yes/no); 2) skin: puffy fingers (yes/no), telangiectasia (yes/no), pitting scars (PS) (yes/no), digital ulcers (DU) (yes/no), gangrene (yes/no), 3) lung: forced vital capacity (FVC) (% of predicted), single-breath diffusion capacity of the lung for carbon monoxide (DLCO[SB]) (% of predicted), SSc lung disease on high-resolution computed tomography (HRCT) (yes/no), maximum oxygen uptake(% of predicted) ; 4) cardiac: tricuspid regurgitation (TR) gradient, left ventricular ejection fraction (LVEF), EA ratio, pericardial effusion (yes/no), proBNP level, PAH (yes as evaluated by right heart catheterization/no or not assessed), arrhythmia (yes/no); 5) renal: history of renal crisis (yes/no), proteinuria (yes/no), 6) musculoskeletal: proximal muscle weakness (yes/no), creatine kinase (CK) level, fingertip-to-palm distance (FTP) of the left and right hand, synovitis (yes/no), friction rubs (yes/no), contractures (yes/no), calcinosis (yes/no), Raynaud phenomenon (RP) (yes/no); 7) gastro-intestinal: albumin level, weight loss >10% (yes/no), dysphagia (yes/no), reflux (yes/no), early satiety (yes/no), vomiting (yes/no), diarrhea (yes/no), intestinal distension (yes/no), constipation (yes/no), fecal incontinence (yes/no), parenteral nutrition (yes/no), history of gastric antral vascular ectasia (GAVE) (yes/no); 8) laboratory findings: CRP level, hemoglobin (Hb) level, ESR, creatinine level. Single imputation was used to replace missing variables (6% of data missing) in clinical variables. Survival (yes/no) at t=5 years since first non-Raynaud phenomenon was determined.

Auto-antibody testing
In a previous study(15), extensive auto-antibody screening in sera of the first 330 patients of the cohort was performed, including ANA (detected by indirect immunofluorescence on HEP-2000 cells) and ENA (measured by fluorescence enzyme-linked immune sorbent assay [FEIA], using Phadia250® system [Thermo Fisher Scientific, Nieuwegein, The Netherlands]). ENA screening included ACA (auto-antigen centromere B), ATA (auto-antigen topoisomerase 1, Scl70 sensitive screening), anti-U1RNP, anti-RNP 70, anti-SSA/Ro, anti-SSB/La, anti-Sm and anti-Jo1. Additionally, anti-RNApIII, anti-Th/To and anti-Ku antibodies were determined for all patients, by a research chemiluminescence immunoassay (CLIA) using the INOVA BioFlash® (Werfen/INOVA, San Diego, USA). In patients with positive ANA but no SSc specific ENA, additionally anti-PmScl and anti-U3RNP were determined.

In 77 patients additionally included in the current study, anti-Th/To and anti-Ku were not routinely determined because of low prevalence (anti-Th/To (0.3%) and anti-Ku (1.3%)), and these antibodies were excluded from the current analysis. Testing regimen for these 77 patients, included ANA and ENA screen, and further testing using Phadia250® for anti-RNApIII, anti-PmScl and anti-U3RNP, when ANA was positive but no SSc specific antibody was detected by ENA.

Cluster analysis methodology
A study flow-chart is shown in Figure 1. We performed unrotated principal component analysis (PCA) with input and standardizing (range of -1 to 1) of solely clinical variables and considered the coordinates of the observations on the retained factorial axes as new variables used for the cluster analysis. As an elbow in the scree plot occurred after 7 obtained factorial axes in both analyses, which explained 36-38 percent of the total variability, these 7 factorial axes were considered and the remaining factors were discarded.

To build homogeneous subgroups of patients, we performed agglomerative hierarchical clustering based on the Ward method. The agglomerative clustering technique starts with every case considered a cluster itself and successively two-by-two merging of clusters until the final merge with all subjects falling into a single category. The metric used to assess proximity between two classes was the Euclidian distance. The process can be plotted as a dendrogram, with horizontal branches representing the combination of two clusters and vertical branches representing the degree of dissimilarity between combined clusters; long distances of the vertical segments indicate large differences between combined clusters.

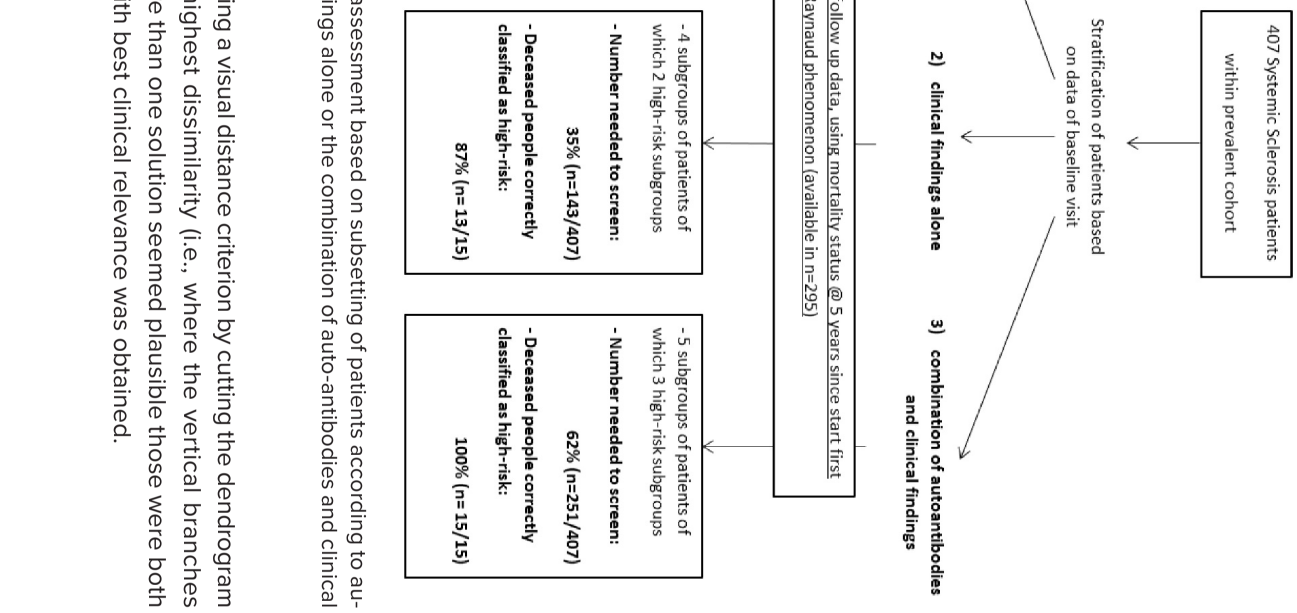

Figure 1 Study flow chart – risk assessment based on subsetting of patients according to auto-antibodies alone, clinical findings alone or the combination of auto-antibodies and clinical findings.

Subgroups were obtained, using a visual distance criterion by cutting the dendrogram horizontally at the level of highest dissimilarity (i.e., where the vertical branches were the longest). When more than one solution seemed plausible those were both assessed and the solution with best clinical relevance was obtained.

Table 1 Baseline characteristics of patients with specific auto-antibodies for Systemic Sclerosis

	CCISS cohort n=407	**ATA*** n=96	**ACA*** n= 145	**RNApIII*** n=21	**U3RNP** n=14	**PmScl*** n=21	**ANA- ENA-** n=16
Survival							
mortality**, % of patients (n)	5.1 (15)	11.4 (8)	3.1 (3)	5.6 (1)	0 (0)	0 (0)	15.4 (2)
Demographic							
age, mean [yrs.] ±SD	55.0±14.4	52.2±14.6	57.2±13.2	64.7±11.5	50.8±13.9	53.5±14.3	53.5±15.5
female sex, % of patients (n)	81.3 (331)	68.8 (66)	89.7 (130)	100 (21)	78.6 (11)	85.7 (18)	62.5 (10)
Caucasians, % of patients (n)	79.3 (315)	68.8 (64)	85.7 (120)	90.0 (18)	57.1 (8)	90.5 (19)	75.0 (12)
Disease specific							
dcSSc, % of patients (n)	23.6 (96)	47.9 (46)	2.8 (4)	38.1 (8)	28.6 (4)	33.3 (7)	37.5 (6)
duration of scleroderma (yr.)							
since onset first Raynaud symptom, median [yrs.] (IQR)	9.7 (3.6-19.2)	6.3 (2.5-13.8)	11.7 (4.9-25.1)	12.4 (4.0-25.2)	6.1 (2.7-12.7)	10.0 (4.2-17.4)	8.4 (3.9-18.9)

Table 1 Baseline characteristics of patients with specific auto-antibodies for Systemic Sclerosis *(continued)*

	CCISS cohort n=407	**ATA*** n=96	**ACA*** n= 145	**RNApIII*** n=21	**U3RNP** n=14	**PmScl*** n=21	**ANA- ENA-** n=16
since onset first non-Raynaud symptom, median [yrs.] (IQR)	4.1 (1.3-10.6)	2.9 (0.7-9.3)	4.0 (1.3-10.6)	4.0 (2.8-11.9)	5.3 (1.3-10.5)	5.8 (2.5-10.7)	3.3 (1.5-8.9)
Skin							
modified Rodnan Skin Score, median (IQR)	4.0 (2.0-6.0)	6.0 (2.0-13.0)	2.0 (0.0-4.0)	4 (1.5-20.0)	8.0 (2.0-9.0)	3.0 (0.0-6.0)	3.5 (0.0-7.0)
Lungs							
FVC, mean [% of predicted] ±SD	100.2±22.8	90.0±22.2	110.8±17.5	110.3±22.3	92.9±28.0	96.3±21.0	89.1±28.0
DLCO, mean [% of predicted] ±SD	64.1±17.6	61.0±16.9	69.4±16.8	62.8±11.2	70.0±23.4	59.0±14.3	65.1±22.0
Lung involvement on HRCT, % of patients (n)	53.6 (218)	72.9 (70)	28.3 (41)	76.2 (16)	57.1 (8)	71.4 (15)	56.3 (9)
Heart							
LVEF, mean±SD	63.5±7.9	61.9±7.6	62.7±7.5	60.5±8.8	65.4±8.7	60.3±6.7	64.2±6.6

Table 1 Baseline characteristics of patients with specific auto-antibodies for Systemic Sclerosis *(continued)*

	CCISS cohort n=407	**ATA*** n=96	**ACA*** n= 145	**RNApIII*** n=21	**U3RNP** n=14	**PmScl*** n=21	**ANA- ENA-** n=16
TR gradient, mean±SD	24.6±10.0	25.1±9.7	23.8±10.0	22.3±7.9	22.6±8.1	21.8±7.6	22.9±6.8
PAH, % of patients (n)	5.9 (24)	5.2 (5)	6.2 (9)	4.8 (1)	0 (0)	4.8 (1)	0 (0)
GI symptoms							
dysphagia, % of patients (n)	44.0 (179)	41.7 (40)	50.3 (73)	57.1 (12)	28.6 (4)	33.3 (7)	43.8 (7)
reflux, % of patients (n)	60.9 (248)	63.5 (61)	61.4 (89)	85.7 (18)	64.3 (9)	42.9 (9)	50.0 (8)
GAVE, % of patients (n)	2.0 (8)	0 (0)	2.1 (3)	0 (0)	0 (0)	4.8 (1)	0 (0)
constipation, % of patients (n)	17.0 (69)	12.5 (12)	20.7 (30)	23.8 (5)	21.4 (3)	14.3 (3)	25.0 (4)
diarrhea, % of patients (n)	15.0 (61)	7.3 (7)	17.2 (25)	14.3 (3)	42.9 (6)	9.5 (2)	6.3 (1)
Renal							
Previous renal crisis, % of patients (n)	3.9 (16)	5.2 (5)	0.7 (1)	14.3 (3)	7.1 (1)	19.0 (4)	0 (0)

Table 1 Baseline characteristics of patients with specific auto-antibodies for Systemic Sclerosis *(continued)*

	CCISS cohort n=407	**ATA*** n=96	**ACA*** n= 145	**RNApIII*** n=21	**U3RNP** n=14	**PmScl*** n=21	**ANA- ENA-** n=16
Peripheral vasculopathy							
Raynaud's phenomenon, % of patients (n)	98.8 (402)	96.9 (93)	98.6 (143)	100 (21)	100 (14)	100 (21)	100 (16)
Pitting scars, % of patients (n)	43.2 (176)	49.0 (47)	38.6 (56)	38.1 (8)	50.0 (7)	61.9 (13)	12.5 (2)
Digital ulcers, % of patients (n)	22.6 (92)	24.0 (23)	26.2 (38)	14.3(3)	21.4 (3)	14.3 (3)	0 (0)

CCISS cohort; Combined Care In Systemic Sclerosis cohort, ANA; anti-nuclear antibodies, ENA; extractable nuclear antibodies, ATA; anti-topoisomerase I antibodies, ACA; anti-centromere antibodies, RNApIII; ribonucleic acid polymerase III, U3RNP; anti-fibrillarin, PmScl; polymyositis scleroderma antibody, GAVE; gastric antral vascular ectasia

* 5 ATA patients, 8 ACA patients, 1 RNApIII patients and 6 PmScl patients were excluded from subgroups in this table because of prevalent auto-antibodies in >1 SSc specific auto-antibody group

**mortality in patients with available follow-up data of at least 5 years since first non-Raynaud phenomenon

This process was performed using demographic and clinical variables, excluding auto-antibody and survival data. Next, this process was repeated, with additional inclusion of 6 variables for disease-specific auto-antibody status (ATA, ACA, RNApIII, U3RNP, PmScl status [positive/negative]).

Clinical relevance of subgroups was assessed by investigating clinical characteristics. Disease-risk was assessed by evaluating subgroup specific mortality. Subgroups were considered to reflect high-risk disease when mortality rates were equal to or higher than the cohort mortality rate.

Statistical analyses

All statistical analyses were performed using IBM SPSS Statistics 23. Subgroup characteristics were tested against cohort values, testing frequencies, medians and means using binomial (1-sided), Wilcoxon-signed rank tests (2-sided) or one-sample T tests (2-sided) as appropriate, p-values ≤0.05 were considered statistically relevant.

Results

Baseline characteristics

Baseline characteristics are shown in Table 1. Of 407 patients included, data on auto-antibody profile were available in 396 patients. Mean age was 55.0±14.4 years, and 81.3% (n=331) of patients were female. Median disease duration since onset of first Raynaud symptom was 9.7 years (IQR 3.6 to 19.2 years) and since onset of first non-Raynaud 4.1 years (IQR 1.3 to 10.6 years). Twenty-three percent (n=96) of patients had dcSSc, mean DLCO was 64±17% of predicted, 23% (n=92) had digital ulcers and 4% (16) of patients had a history of renal crisis. Median available follow-up time was 3.8 years (IQR 2.0-5.8 years), with 5-year survival status since first non-Raynaud phenomenon available in 72% (n=295). Of the remaining patients, 27% (n=109) had follow-up shorter than 5 years since onset first non-Raynaud phenomenon and in 1% (n=3) follow-up status was missing.

Auto-antibody prevalences were: ACA 38% (n=153/399), ATA 25% (n=101/401), RNApIII 6% (n=22/398), U3RNP 4% (n=14/397) for and PmScl 7% (n=27/397). Four percent (n=17/402) of patients were both ANA and ENA negative. Co-occurrence of disease-specific auto-antibodies was found in 10 patients (ATA/ACA overlap n=3 [ACA weakly positive n=1; ATA weakly positive n=1; both weakly positive n=1] , ACA/PmScl overlap n=4 [ACA weakly positive n=1], ATA/PmScl overlap n=2 [PmScl weakly positive n=1], ACA/RNApIII overlap n=1 [RNApIII weakly positive]).

Stratification of patients based on clinical variables

Using solely clinical variables, factor axes of the principal component analysis, included for the cluster process, explained 38% of variance in the data. Hierarchical clustering of these factors was compatible with a 4-cluster solution (Figure 2). The clinical phenotype of the patients in these 4 subgroups is shown in Table 2.

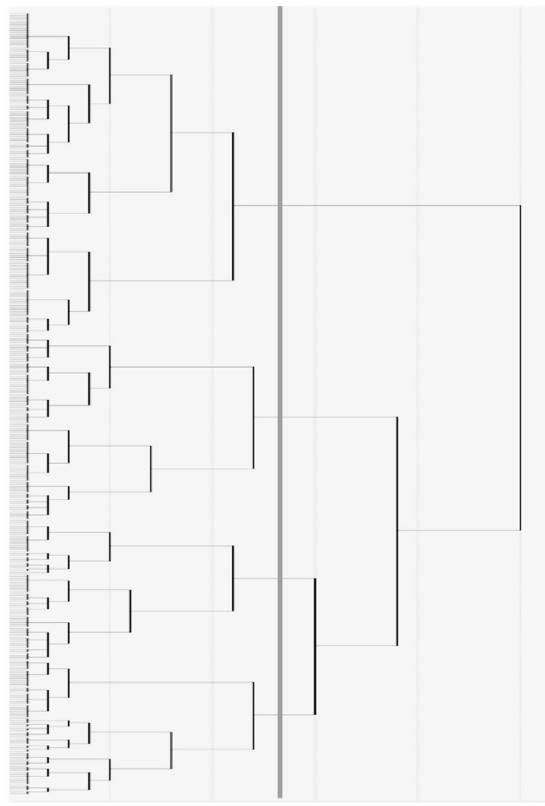

Figure 2 Dendrogram of cluster analysis of Systemic Sclerosis patients using solely clinical variables Cluster process was done by Wards method, using Euclidean distance on standardized variables (range -1 to 1) of scores on the first 7 factors obtained by principal component analysis on 52 clinical variables (including demographic, skin, lung, cardiac, gastro-intestinal, renal and laboratory variables). The full dendrogram displays progressive clustering of subjects. The bold horizontal line marks the level of truncation, resulting in 4 obtained subgroups of patients.

Subgroup 1 represented a subgroup with more men (male 76%, n=53, p<0.001), more dcSSc (57.1%, n=40, p<0.001), higher mRSS scores (mean 10.3±10.6 SD, p<0.001), and more renal crisis (16%, n=11, p<0.001). As seen from FVC (mean 91%, p<0.001), DLCO (mean 59%, p<0.018) and lung involvement on HRCT (31%, p=0.008) ILD was present, but less frequent within this subgroup; also GI involvement was less frequent. Median follow-up time was 3.7 years (IQR 1.5-5.9). Five-year follow-up data since first non-Raynaud phenomenon were available in 76% (n=53), showing high-risk disease, illustrated by a mortality rate of 17% (p<0.001).

Table 2. Clinical characteristics and autoantibody prevalences within Systemic Sclerosis subgroups obtained by cluster analysis using solely clinical variables

subgroup	**1** (n=70)	p*	**2** (n=73)	p*	**3** (n=97)	p*	**4** (n=167)	p*
Survival								
mortality, % of patients (n)**	17.0 (9)	**0.001**	7.0 (4)	**0.331**	0.0 (0)	**0.017**	1.9 (2)	**0.085**
Demographic								
age, mean [yrs.] ±SD	55.9±14.6	0.615	55.6±15.6	0.634	56.9±12.2	0.129	53.3±14.8	0.150
female sex, % of patients (n)	24.3 (17)	**<0.001**	95.9 (70)	**<0.001**	89.7 (87)	**0.018**	94.0 (157)	**<0.001**
Caucasians, % of patients (n)	78.3 (54)	0.373	56.5 (39)	**<0.001**	88.4 (84)	**0.045**	84.1 (138)	0.167
Disease specific								
dcSSc, % of patients (n)	57.1 (40)	**<0.001**	30.1 (22)	0.121	12.4 (12)	**0.004**	13.2 (22)	**0.001**
duration of scleroderma (yr.)								
since onset first Raynaud symptom, median [yrs.] (IQR)	5.2 (1.3-12.3)	0.086	8.3 (3.5-19.6)	0.154	19.8 (10.1-35.9)	**<0.001**	6.9 (3.1-14.7)	0.164
since onset first non-Raynaud symptom, median [yrs.] (IQR)	2.7 (0.8-8.5)	0.746	6.1 (2.4-12.4)	**<0.001**	10.6 (3.0-19.1)	**<0.001**	2.5 (0.9-5.6)	**0.011**
Skin								
modified Rodnan Skin Score, median (IQR)	6 (2-18)	**<0.001**	4 (0.5-7.95)	0.325	4 (2-6)	0.764	2 (0-4)	**<0.001**
Lungs								

Table 2. Clinical characteristics and autoantibody prevalences within Systemic Sclerosis subgroups obtained by cluster analysis using solely clinical variables *(continued)*

subgroup	**1** (n=70)	p*	**2** (n=73)	p*	**3** (n=97)	p*	**4** (n=167)	p*
FVC, mean [% of predicted] ±SD	91.4±18.8	**<0.001**	82.7±23.9	**<0.001**	104.2±21.0	0.064	108.8±19.3	**<0.001**
DLCO, mean [% of predicted] ±SD	58.5±19.4	**0.018**	47.3±13.1	**<0.001**	66.1±13.9	0.163	72.6±14.1	**<0.001**
Lung involvement on HRCT, % of patients (n)	31.4 (22)	**0.008**	67.1 (49)	**0.013**	49.5 (48)	0.238	43.7 (73)	**0.007**
Heart								
LVEF, mean±SD	59.5±7.9	**<0.001**	65.7±9.2	**0.046**	62.0±7.0	**0.040**	62.8±7.1	0.218
TR gradient, mean±SD	26.0±11.7	0.321	32.8±13.5	**<0.001**	22.8±7.2	**0.021**	21.3±6.0	**<0.001**
PAH, % of patients (n)	4.3 (3)	0.402	21.9 (16)	**0.001**	2.1 (2)	0.070	1.8 (3)	**0.010**
GI symptoms								
dysphagia, % of patients (n)	25.7 (18)	**0.001**	42.5 (31)	0.444	81.4 (79)	**<0.001**	30.5 (51)	**<0.001**
reflux, % of patients (n)	57.1 (40)	0.609	64.4 (47)	0.315	89.7 (87)	**<0.001**	44.3 (74)	**<0.001**
GAVE,% of patients (n)	0 (0)	**<0.001**	5.5 (4)	**<0.001**	3.1 (4)	**0.001**	0.6 (1)	0.284
constipation, % of patients (n)	10.0 (7)	0.074	13.7 (10)	0.284	32.0 (31)	**<0.001**	12.6 (21)	0.074
diarrhea, % of patients (n)	4.3 (3)	**0.004**	12.3 (9)	0.328	35.1 (34)	**<0.001**	9.0 (15)	**0.015**
Renal								

Table 2. Clinical characteristics and autoantibody prevalences within Systemic Sclerosis subgroups obtained by cluster analysis using solely clinical variables *(continued)*

subgroup	**1** (n=70)	p*	**2** (n=73)	p*	**3** (n=97)	p*	**4** (n=167)	p*
Previous renal crisis, % of patients (n)	15.7 (11)	**<0.001**	1.4 (1)	0.217	2.1 (2)	0.266	1.2 (2)	**0.040**
Peripheral vasculopathy								
Raynaud's phenomenon, % of patients (n)	95.7 (67)	0.052	100 (73)	0.414	97.9 (95)	0.325	100 (167)	0.133
Pitting scars, % of patients (n)	44.3 (31)	0.473	54.8 (40)	**0.030**	52.6 (51)	**0.040**	32.3 (100)	**0.003**
Digital ulcers, % of patients (n)	17.1 (12)	0.172	27.4 (20)	0.198	32.0 (31)	**0.022**	17.4 (29)	0.060
Auto-antibodies								
ATA, % of patients (n)[1]	49.3 (34)	**<0.001**	30.6 (22)	0.170	10.4 (10)	**<0.001**	21.3 (35)	0.161
ACA, % of patients (n)[2]	11.6 (8)	**<0.001**	25.4 (18)	**0.017**	55.2 (53)	**<0.001**	45.4 (74)	**0.032**
RNApIII, % of patients (n)[3]	5.8 (4)	0.601	1.4 (1)	0.068	7.4 (7)	0.344	6.1 (10)	0.517
U3RNP, % of patients (n)[4]	1.4 (1)	0.232	7.0 (5)	0.155	3.2 (3)	0.470	3.1 (5)	0.368
PmScl % of patients (n)[5]	7.5 (5)	0.548	2.8 (2)	0.112	8.4 (8)	0.362	7.4 (12)	0.483

[1] unknown in 6; [2] unknown in 8; [3] unknown in 9; [4] unknown in 10; [5] unknown in 10 patients

* p-values are based on one-sample testing against cohort means/prevalences shown in Table 1

**mortality in patients with available follow-up data of at least 5 years since first non-Raynaud phenomenon

(subgroup 1 n=53; subgroup 2 n=57; subgroup 3 n=78; subgroup 4 n=104)

Subgroup 2 consisted of patients often female (96%, n=70, p=<0.001) and less often Caucasian (57%, n=39, p=<0.001). Time since onset of the first non-Raynaud was relatively long with a mean of 6.1 years (p<0.001). PAH (22%, n=16, p=0.001), GAVE (6%, n=4, p<0.001), ILD (median FVC 83% [p<0.001], median DLCO 47% [p=<0.001], lung involvement on HRCT 67% [p<0.013]), pitting scars (55%, n=40, p=0.030) were frequent. Median follow-up time was 4.3 years (IQR 2.5-6.8), five-year follow-up data since first onset of a non-Raynaud phenomenon in this group was available in 76% (n=57), showing high-risk disease, illustrated by a mortality rate of 7% (p=<0.001).

Subgroup 3 consisted of predominantly female (90%, =0.018), Caucasian (88%, p=0.045), lcSSc (74.2%, p<0.001) patients with frequent GI symptoms (dysphagia 81% [n=79], reflux 90% [n=87], constipation 32% [n=31], diarrhea 35% [n=34], all p<0.001). Peripheral vasculopathy was frequent (pitting scars 53% [p=0.04], digital ulcers 32% [p=0.022]). Median FU time was 3.9 years (IQR 2.4-5.2), however in 20% (n=19) 5-year survival since onset of first non-Raynaud was not available. Although disease duration since onset first Raynaud (median 20 years) and since onset first non-Raynaud phenomenon (median 11 year) was long, no mortality was reported, indicating low disease-risk (p=<0.001).

Subgroup 4 predominantly consisted of females (94.0%, n=157) with lcSSc (dcSSc 13%, n=22). Median time since first non-Raynaud was short with 2.5 years (p=0.011). Lungs were less affected compared to the total cohort (mean FVC 109% [p<0.001], mean DLCO 73% [p<0.001], lung involvement 44% [p=0.007]) and GI symptoms occurred less often (dysphagia 31% [p<0.001], reflux 44% [p<0.001], diarrhea 9% [p=0.015]). Median follow-up time in this group was 3.6 years (IQR 1.9-5.3) and 5-year follow-up since first non-Raynaud was available in 36% (n=60); of these 60 patients, one died (2%; p <0.001).

Although auto-antibodies were not taken into account in the subgroup process, auto-antibodies were not distributed evenly. ATA was dominant in subgroup 1 (49%[n=34], p=0.001) and subgroup 2 (31%[n=22], p=0.017). ACA was the most prevalent auto-antibody in subgroups 3 (55% [n=53], p<0.001) and 4 (45% [n=74], p=0.032). Prevalences of RNApII, U3RNP and PmScl within subgroups did not significantly differ from the population means within each subgroup. Notably, in subgroup 2 the number of ATA positive patients (30.6%, n=22) was almost equal to the number of ACA positive patients (25.4%, n=18). 55%(n=56) of ATA patients were stratified to subgroup 1 and 2, and 45% (n=45)to subgroup 3 and 4.

Table 3. Clinical characteristics and auto-antibody prevalences within Systemic Sclerosis subgroups obtained by cluster analysis using clinical variables and disease specific auto-antibody status

	subgroup									
	1 (n=73)	**p***	**2** (n=61)	**p***	**3** (n=91)	**p***	**4** (n=85)	**p***	**5** (n=97)	**p***
Survival										
mortality**, % of patients (n)	10.0 (6)	0.085	9.1 (4)	0.185	0.0 (0)	**0.026**	0.0 (0)	**0.066**	7.2 (5)	0.279
Demographic										
age, mean [yrs.] ±SD	54.3±13.2	0.637	52.2±17.2	0.212	58.0±11.4	**0.013**	46.0±13.5	**<0.001**	62.5±11.6	**<0.001**
female sex, % of patients (n)	38.4 (28)	**<0.001**	95.1 (58)	**0.002**	89.0 (81)	**0.034**	84.7 (72)	0.258	94.8 (92)	**<0.001**
Caucasians, % of patients (n)	77.1 (54)	0.163	48.3 (28)	**<0.001**	84.3 (75)	0.278	82.1 (69)	0.394	92.7 (89)	0.001
Disease specific										
dcSSc, % of patients (n)	57.5 (42)	**<0.001**	26.2 (16)	0.361	9.9 (9)	**0.001**	8.2 (7)	**<0.001**	22.7 (22)	0.471
duration of scleroderma (yr.)										
since onset first Raynaud symptom, median [yrs.] (IQR)	11.5 (3.2-20.1)	0.099	7.9 (2.9-14.1)	0.407	15.5 (7.0-28.2)	**<0.001**	6.4 (2.8-13.4)	0.095	9.5 (3.4-19.1)	0.212

Table 3. Clinical characteristics and auto-antibody prevalences within Systemic Sclerosis subgroups obtained by cluster analysis using clinical variables and disease specific auto-antibody status *(continued)*

	subgroup									
	1 (n=73)	**p***	**2** (n=61)	**p***	**3** (n=91)	**p***	**4** (n=85)	**p***	**5** (n=97)	**p***
since onset first non-Raynaud symptom, median [yrs.] (IQR)	7.2 (1.3-14.6)	**<0.001**	4.7 (1.3-10.3)	**0.015**	6.1 (2.5-15.0)	**<0.001**	2.0 (0.5-5.8)	**0.034**	3.1 (1.2-7.3)	0.883
Skin										
modified Rodnan Skin Score, median (IQR)	6.0 (2.0-17.5)	**<0.001**	4.0 (0-7.45)	0.700	4.0 (1.0-6.0)	0.424	2.0 (0.0-4.0)	**<0.001**	4.0 (1.0-6.0)	0.292
Lungs										
FVC, mean [% of predicted] ±SD	84.9±18.4	**<0.001**	82.9±22.5	**<0.001**	104.8±22.7	**0.044**	105.7±17.5	**0.005**	112.6±18.7	**<0.001**
DLCO, mean [% of predicted] ±SD	55.32±17.4	**<0.001**	49.5±14.5	**<0.001**	65.8±14.8	0.288	73.7±17.0	**<0.001**	69.9±13.4	**<0.001**
Lung involvement on HRCT, % of patients (n)	78.1 (57)	**<0.001**	57.4 (35)	0.323	45.1 (41)	0.063	35.3 (30)	**0.001**	56.7 (55)	0.306
Heart										
LVEF, mean±SD	58.0±7.5	**<0.001**	67.8±9.4	**0.001**	62.5±6.9	0.162	62.0±6.9	0.052	63.3±6.6	0.732
TR gradient, mean±SD	26.2±9.6	0.149	33.9±14.0	**<0.001**	22.5±8.3	0.018	21.2±5.6	**<0.001**	22.2±7.9	**0.004**

Table 3. Clinical characteristics and auto-antibody prevalences within Systemic Sclerosis subgroups obtained by cluster analysis using clinical variables and disease specific auto-antibody status *(continued)*

	subgroup									
	1 (n=73)	**p***	**2** (n=61)	**p***	**3** (n=91)	**p***	**4** (n=85)	**p***	**5** (n=97)	**p***
PAH, % of patients (n)	4.1 (3)	0.369	26.2 (16)	**<0.001**	2.2 (2)	0.090	1.2 (1)	**0.036**	2.1 (2)	0.070
GI symptoms										
dysphagia, % of patients (n)	41.1 (30)	0.353	42.6 (26)	0.467	79.1 (72)	**<0.001**	15.3 (13)	**<0.001**	39.2 (38)	0.197
reflux, % of patients (n)	71.2 (52)	0.044	52.5 (32)	0.112	85.7 (78)	**<0.001**	29.4 (25)	**<0.001**	62.9 (61)	0.386
GAVE,% of patients (n)	0 (0)	-	6.6 (4)	**<0.001**	3.3 (3)	**0.001**	1.2 (1)	0.156	0 (0)	**<0.001**
constipation, % of patients (n)	11.0 (8)	0.107	18.0 (11)	0.467	29.7 (27)	**0.002**	5.9 (5)	**0.002**	18.6 (18)	0.382
diarrhea, % of patients (n)	5.5 (4)	**0.011**	11.5 (7)	0.287	47.3 (43)	**<0.001**	2.4 (2)	**<0.001**	5.2 (5)	**0.002**
Renal										
Previous renal crisis, % of patients (n)	15.1 (11)	**<0.001**	3.3 (2)	0.573	0.0 (0)	**0.027**	0 (0)	**0.034**	3.1 (3)	0.474
Peripheral vasculopathy										
Raynaud's phenomenon, % of patients (n)	97.3 (71)	0.218	100.0 (85)	0.479	97.8 (89)	0.298	100 (0)	0.358	99.0 (96)	0.675

Table 3. Clinical characteristics and auto-antibody prevalences within Systemic Sclerosis subgroups obtained by cluster analysis using clinical variables and disease specific auto-antibody status *(continued)*

	subgroup									
	1 (n=73)	**p***	**2** (n=61)	**p***	**3** (n=91)	**p***	**4** (n=85)	**p***	**5** (n=97)	**p***
Pitting scars, % of patients (n)	61.6 (45)	**<0.001**	54.1 (33)	0.057	40.7 (37)	0.352	45.9 (39)	0.347	22.7 (22)	**<0.001**
Digital ulcers, % of patients (n)	31.5 (23)	0.050	27.9 (17)	0.201	20.9 (19)	0.403	29.4 (25)	0.088	8.2 (8)	**<0.001**
Auto-antibodies										
ATA, % of patients (n)[2]	49.3 (35)	**<0.001**	29.5 (18)	0.249	5.6 (5)	**<0.001**	20.5 (17)	0.207	26.8 (26)	0.378
ACA, % of patients (n)[3]	2.8 (2)	**<0.001**	26.7 (16)	**0.044**	66.3 (59)	**<0.001**	48.8 (40)	**0.030**	37.1 (36)	0.473
RNApIII, % of patients (n)[4]	4.2 (3)	0.377	0.0 (0)	**<0.001**	4.5 (4)	0.386	0.0 (0)	**0.006**	15.5 (15)	**<0.001**
U3RNP, % of patients (n)[5]	2.8 (2)	0.456	5.0 (3)	0.432	5.7 (5)	0.276	3.7 (3)	0.593	1.0 (1)	0.096
PmScl % of patients (n)[5]	11.3 (8)	0.130	5.0 (3)	0.376	5.7 (5)	0.400	13.6 (11)	**0.028**	0.0 (0)	**0.001**

[1] unknown in 6; [2] unknown in 8; [3] unknown in 9; [4] unknown in 10; [5] unknown in 10 patients
* p-values are based on one-sample testing against cohort means/prevalences shown in Table 1
**mortality in patients with available follow-up data of at least 5 years since first non-Raynaud phenomenon (subgroup 1 n=60; subgroup 2 n=44; subgroup 3 n=70; subgroup 4 n=52; subgroup 5 n=69)

Stratification of patients based on clinical variables and disease-specific auto-antibodies

Using clinical variables and additionally, auto-antibody status, factor axes of principal component analysis, included for hierarchical clustering, explained 36% of variance in the data. Hierarchical clustering of these factors was compatible with a 5-cluster solution (Figure 3). Clinical characteristics of the patients in the different subgroups are shown in Table 3.

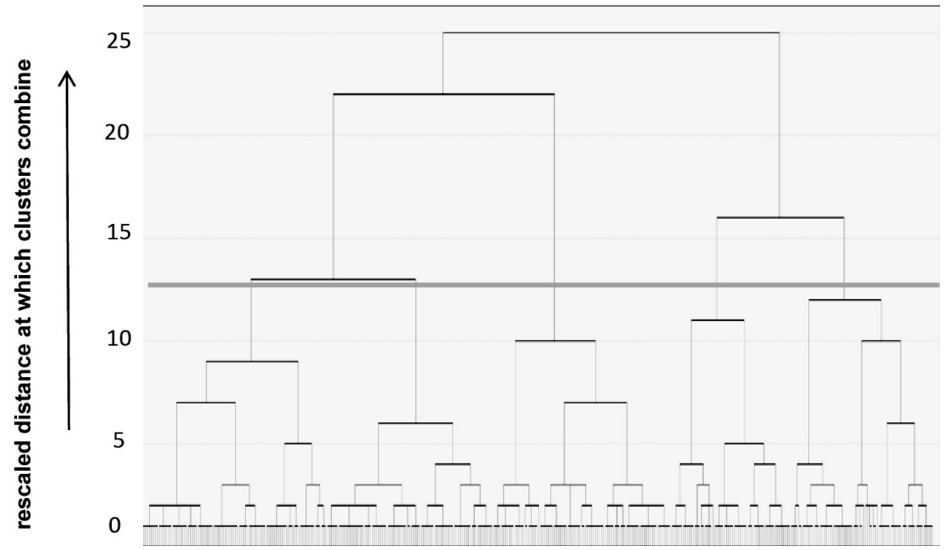

Figure 3 Dendrogram of cluster analysis of Systemic Sclerosis patients using clinical variables and auto-antibodies. Cluster process was done by Wards method, using Euclidean distance on standardized variables (range -1 to 1) of scores on the first 7 factors obtained by principal component analysis on 52 clinical variables (including demographic, skin, lung, cardiac, gastro-intestinal, renal and laboratory variables)and status of anti-topoisomerase I, anti-centromere, anti RNA polymerase III, anti-U3RNP and anti-PmScl antibodies. The full dendrogram displays progressive clustering of subjects. The bold horizontal line marks the level of truncation, resulting in 5 obtained subgroups of patients.

As compared to the cohort, patients in subgroup 1 were less often female (38%, p<0.001), more often had dcSSc (58%, p<0.001), longer disease duration (median 7.2 year since onset first non-Raynaud phenomenon, p<0.001) and more renal crisis (15%, p<0.001). Mortality rate within this subgroup was 10% (p=0.085). In subgroup 2, the frequency of Caucasians was less (48%, p<0.001) and prevalence of dcSSc (43%), PAH (26%, p<0.001) and GAVE (7%, p<0.001) were higher than expected. Disease risk in subgroup 2 was high, with a 9% mortality rate (p=0.185). Subgroup 3 and 4 included patients with low disease-risk (mortality rates both 0%). Subgroup 3 was

characterized by a high frequency of GI involvement and subgroup 4 represented a miscellaneous subgroup. The additional subgroup 5 was characterized by less frequent ILD (mean predicted FVC 113%, p<0.001; mean predicted DLCO 69.9 p<0.001), low TR gradients (mean 22 mmHg, p<0.004) and less frequent vasculopathy (pitting scars 16% [p<0.001], digital ulcers 6% [p<0.001]). However, it was also a high-risk subgroup with a 7.2% mortality rate (p=0.279).

Of disease-specific auto-antibodies, ATA was dominant in both the high-risk subgroups (subgroup 1 [49%, p=<0.001] and subgroup 2 [30%. p=0.249]) and ACA was dominant in the low-risk subgroups (subgroup 3 [66%, p=<0.001] and subgroup 4 [49%, p=0.030]). In the additional subgroup 5 ACA was also the most frequent auto-antibody (37%, p=0.473). This subgroup was additionally characterized by a high prevalence of RNApIII auto-antibodies (16%, p<0.001). 78% (n=79) of ATA patients were stratified to subgroup 1,2 or 5, and 22%(n=22) to subgroup 3 and 4.

Value of derived subgroups in risk-stratification

To value derived subgroups, the amount of patients clustered into high-risk disease subgroups were compared between stratification based on auto-antibody status alone, stratification based on clinical variables and stratification based on both clinical variables and auto-antibody status. Based on auto-antibody status alone 33% (n=133/407 [ATA+, RNApIII+, ANA-ENA-]) of patients were considered high-risk including 80% (n=12/15) of the deceased patients. Based on clinical variables alone, 35% (n=143/407) of patients were classified as high-risk, which included 87% (n=13/15) of the deceased patients. Combining clinical data with data on auto-antibodies resulted in 57% (n=231/407) of patients being classified as high-risk, with all deceased included. Clinical characteristics that advocate specific diagnostic tests for follow-up including pulmonary involvement (as reflected by HRCT), renal crisis and pulmonary arterial hypertension were present in all the different subgroups, either using auto-antibodies, clinical or combined data for stratification.

Discussion

With this study we aimed at assessing the additional value of auto-antibodies as markers for severe disease course in SSc, in the clinical setting. We show that when auto-antibodies are taken into account, the percentage of patients with actual severe disease course correctly identified as such increases. However, it should be noted, that risk-stratification is still far from perfect as demonstrated by the increasing number of patients stratified in high-risk subgroups.

Of note, while ATA is the antibody most prevalent in the high-risk subgroups, the number of ATA positive patients among low-risk subgroups is considerable. Clustering

based on both clinical characteristics and auto-antibody status, resulted in 22% (n=22/101) of ATA patients being classified as low-risk. Similarly, 35% (n=54/153) of ACA patients seem prone to high-risk (Table 3).

Based on these findings we conclude that estimating prognosis for the individual patient based on auto-antibody status alone, as is suggested for early disease (7, 11), is imprecise and as such inappropriate. Conform these findings, Iniesta Arandia et al. showed survival amongst patients with RNApIII, ATA and ACA antibodies is similar, although distinctive clinical phenotypes among immunologic profiles exist(16). Likewise, the studies of Kranenburg et al. 2016 and Cottrell et al. 2014 demonstrate that prognosis cannot solely be estimated based on auto-antibody status, but assessment of clinical features such as skin is meaningful (10, 17).

Nevertheless, auto-antibodies are correlated with and do predict distinct clinical phenotypes(7), as is also shown by improved detection of lung involvement (from n=71/218 to n=147/218), PAH (from n=19/24 to n=21/24) and renal crisis (from n=12/16 to n=16/16), when shifting from clinical subgrouping to combined auto-antibody and clinical subgrouping. Given the clear but weak association between auto-antibody status we hypothesize that other auto-antibody characteristics are of relevance for auto-antibody pathogenicity as has been described in other auto-immune diseases. For instance, in rheumatoid arthritis it has been shown that an immune response covering a broader selection of isotypes is associated with risk for future radiographic damage(18). The MPO-ANCA aa–447-459 –epitope in vasculitis is associated with active disease(19) and sialylation levels of anti-proteinase 3 antibodies are associated with disease activity in Wegener's disease(20). Further investigation of auto-antibody characteristics such as fine-specificity, isotype prevalences, Fc-glycosylation and titer fluctuations and their usefulness for prediction of high-risk disease in SSc, is therefore warranted. In small groups of SSc patients, such studies seem promising. For example, it has been shown that ATA titers correlate with skin involvement (21, 22) and low or high RNApIII intensity on immunoblot assay is associated with clinically distinct phenotypes in SSc (23).

This study has some limitations which should be taken into account. Although we included a relatively large number of patients prospectively, with a low percentage of data missing, varying disease durations at baseline together with a limited follow-up time available in some patients implicates that data interpretation should be performed with caution. Additionally, although mortality did not differ much from mortality in other prevalent cohorts (3, 24), the general low mortality risk makes prediction of mortality more difficult. Nevertheless, our main focus was to evaluate the additional value of auto-antibodies next to clinical characteristics, not identifying distinguishable clinical phenotypes. Disease duration was accounted for by taking this factor into account in both the clinical principal component analysis and the

analysis including antibodies as well. In addition, for assessing risk only patients with five years follow-up since first non-Raynaud symptom available were taken into account. Finally, evaluating disease duration according to antibody status within the different subgroups identified in the clinical model did not show any significant differences in disease duration for ATA+ vs ATA- and ACA+ vs ACA- (data not shown).

In summary, using data from our well described, prospective SSc cohort with annual, complete and comprehensive clinical and auto-antibody data available, we subsequently performed cluster analyses with and without inclusion of auto-antibody status and show that auto-antibodies are of additional value in risk-stratification and clinical subsetting in SSc. This underlines the hypothesis that auto-antibodies contribute to disease pathogenesis. However, the additional value is limited, which is demonstrated by the fact that albeit all high-risk patients are correctly identified by taking auto-antibodies into account, the number of patients wrongly identified as possibly high-risk increases by 66%, from 130 to 216. Our findings confirm that not all ATA-positive patients have worse prognosis and as such additional biomarkers are needed to guide clinical follow-up in SSc. Further research in auto-antibody characteristics as a biomarker in prevalent disease and the value of auto-antibody status for risk-assessment in incident cases is warranted.

References

1. Allanore Y, Simms R, Distler O, Trojanowska M, Pope J, Denton CP, et al. Systemic sclerosis. Nature reviews Disease primers. 2015;1:15002.
2. Muangchan C, Canadian Scleroderma Research G, Baron M, Pope J. The 15% rule in scleroderma: the frequency of severe organ complications in systemic sclerosis. A systematic review. J Rheumatol. 2013;40(9):1545-56.
3. Hao Y, Hudson M, Baron M, Carreira P, Stevens W, Rabusa C, et al. Early Mortality in a Multinational Systemic Sclerosis Inception Cohort. Arthritis & rheumatology. 2017;69(5):1067-77.
4. Barsotti S, Bruni C, Orlandi M, Della Rossa A, Marasco E, Codullo V, et al. One year in review 2017: systemic sclerosis. Clin Exp Rheumatol. 2017;35 Suppl 106(4):3-20.
5. Khanna D, Denton CP. Evidence-based management of rapidly progressing systemic sclerosis. Best Pract Res Clin Rheumatol. 2010;24(3):387-400.
6. LeRoy EC, Medsger TA, Jr. Criteria for the classification of early systemic sclerosis. J Rheumatol. 2001;28(7):1573-6.
7. Steen VD. Autoantibodies in systemic sclerosis. Semin Arthritis Rheum. 2005;35(1):35-42.
8. Morrisroe K, Huq M, Stevens W, Rabusa C, Proudman SM, Nikpour M, et al. Risk factors for development of pulmonary arterial hypertension in Australian systemic sclerosis patients: results from a large multicenter cohort study. BMC Pulm Med. 2016;16(1):134.
9. Coghlan JG, Denton CP, Grünig E, Bonderman D, Distler O, Khanna D, et al. Evidence-based detection of pulmonary arterial hypertension in systemic sclerosis: the DETECT study. Ann Rheum Dis. 2014;73(7):1340-9.
10. Kranenburg P, van den Hombergh WM, Knaapen-Hans HK, van den Hoogen FH, Fransen J, Vonk MC. Survival and organ involvement in patients with limited cutaneous systemic sclerosis and anti-topoisomerase-I antibodies: determined by skin subtype or auto-antibody subtype? A long-term follow-up study. Rheumatology (Oxford). 2016;55(11):2001-8.
11. Kuwana M. Circulating Anti-Nuclear Antibodies in Systemic Sclerosis: Utility in Diagnosis and Disease Subsetting. Journal of Nippon Medical School = Nippon Ika Daigaku zasshi. 2017;84(2):56-63.
12. Wangkaew S, Euathrongchit J, Wattanawittawas P, Kasitanon N, Louthrenoo W. Incidence and predictors of interstitial lung disease (ILD) in Thai patients with early systemic sclerosis: Inception cohort study. Mod Rheumatol. 2016;26(4):588-93.
13. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/ European League against Rheumatism collaborative initiative. Arthritis Rheum. 2013;65(11):2737-47.
14. Meijs J, Schouffoer AA, Ajmone Marsan N, Kroft LJ, Stijnen T, Ninaber MK, et al. Therapeutic and diagnostic outcomes of a standardised, comprehensive care pathway for patients with systemic sclerosis. RMD open. 2016;2(1):e000159.
15. Markusse IM, J.; de Boer, B.; Bakker, J.; Schippers, P.; Schouffoer, A.; Ajmone Marsan, N.; Kroft, L.; Ninaber, M.; Huizinga, T.; de Vries-Bouwstra, J. The Additive Value of Nailfold Videocapillaroscopy Patterns to Disease-Specific Autoantibodies in Discrimination of Patients with Systemic Sclerosis at Risk for Severe Organ Involvement. Ann Rheum Dis. 2015;74(Supp 2):597.

16. Iniesta Arandia N, Simeon-Aznar CP, Guillen Del Castillo A, Colunga Arguelles D, Rubio-Rivas M, Trapiella Martinez L, et al. Influence of antibody profile in clinical features and prognosis in a cohort of Spanish patients with systemic sclerosis. Clin Exp Rheumatol. 2017;35 Suppl 106(4):98-105.
17. Cottrell TR, Wise RA, Wigley FM, Boin F. The degree of skin involvement identifies distinct lung disease outcomes and survival in systemic sclerosis. Ann Rheum Dis. 2014;73(6):1060-6.
18. van der Woude D, Syversen SW, van der Voort EI, Verpoort KN, Goll GL, van der Linden MP, et al. The ACPA isotype profile reflects long-term radiographic progression in rheumatoid arthritis. Ann Rheum Dis. 2010;69(6):1110-6.
19. Roth AJ, Ooi JD, Hess JJ, van Timmeren MM, Berg EA, Poulton CE, et al. Epitope specificity determines pathogenicity and detectability in ANCA-associated vasculitis. J Clin Invest. 2013;123(4):1773-83.
20. Espy C, Morelle W, Kavian N, Grange P, Goulvestre C, Viallon V, et al. Sialylation levels of anti-proteinase 3 antibodies are associated with the activity of granulomatosis with polyangiitis (Wegener's). Arthritis Rheum. 2011;63(7):2105-15.
21. Hu PQ, Fertig N, Medsger TA, Jr., Wright TM. Correlation of serum anti-DNA topoisomerase I antibody levels with disease severity and activity in systemic sclerosis. Arthritis Rheum. 2003;48(5):1363-73.
22. Hasegawa M, Imura-Kumada S, Matsushita T, Hamaguchi Y, Fujimoto M, Takehara K. Anti-topoisomerase I antibody levels as serum markers of skin sclerosis in systemic sclerosis. J Dermatol. 2013;40(2):89-93.
23. Patterson KA, Roberts-Thomson PJ, Lester S, Tan JA, Hakendorf P, Rischmueller M, et al. Interpretation of an Extended Autoantibody Profile in a Well-Characterized Australian Systemic Sclerosis (Scleroderma) Cohort Using Principal Components Analysis. Arthritis Rheumatol. 2015;67(12):3234-44.
24. Elhai M, Meune C, Boubaya M, Avouac J, Hachulla E, Balbir-Gurman A, et al. Mapping and predicting mortality from systemic sclerosis. Ann Rheum Dis. 2017;76(11):1897-905.

4

Auto-antibodies and cancer in Systemic Sclerosis

M. Boonstra, J.K. de Vries-Bouwstra, T.W.J. Huizinga

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Dear Editor,

With interest we have read the article by Bernal-Bello et al., associating Pm/Scl antibodies with a higher risk of cancer in Systemic Sclerosis (SSc) (1). We appreciate the research this group performed as early detection of malignancies in Systemic Sclerosis patients is important in daily clinical practice.

Bernal-Bello et al. retrospectively analysed data of 432 consecutive SSc patients and found a cancer prevalence of 12.2% (n=53) with decreased survival for SSc patients with cancer. Pm/Scl antibody prevalence of 20.7% (n=6/29) amongst SSc patients with cancer diagnosis compared with 7.7% (n=19/247) amongst SSc patients not being diagnosed with cancer is shown, together with increased cancer prevalence amongst Pm/Scl patients (24%, n=6/25). The authors conclude that Pm/Scl antibodies are associated with malignancies in SSc and patients being Pm/Scl antibody positive might benefit from comprehensive cancer screening (1). The authors acknowledge the limitations of their design and suggest that their data should be replicated in other cohorts. For example, not all included patients were serologically evaluated for Pm/Scl positivity.

We took advantage of our prospective SSc cohort including all patients that participate in the multidisciplinary day-care program for SSc at the Leiden University Medical Center (LUMC) (2) in order to evaluate the association between auto-antibodies and cancer diagnosis. We re-evaluated 46 SSc patients with a history of malignancy amongst 305 SSc patients with recent follow-up and at least 2 visits to our care pathway available. Sera of 280 patients were tested for ANA screen, ENA (anti-SSA, anti-SSB, anti-centromere [ACA], anti-topoisomerase [ATA], anti-RNP70, anti-U1RNP, anti-Smith, anti-Jo), anti-U3RNP (fibrillarin), anti-Pm/Scl, anti-RNA polymerase III (RNApIII), anti-Th/To and anti-Ku. In the remaining 25 patients, Th/To and Ku was not determined and RNApIII, U3RNP and Pm/Scl status was only determined if ANA screening was positive and ENA screening did not reveal any disease-specific auto-antibodies, based on the result in the first 280 patients. Prevalence of ACA was 38.0% (n=116), ATA 25.9% (n=79), RNApIII 6.6% (n=20), U3RNP 4.3% (n=13), U1RNP 9.2% (n=28), Pm/Scl 6.9% (n=21), anti-ThTo 1.6% (n=5), anti-Ku 2.0% (n=6), ANA-ENA- 4.3% (n=13), ANA+/ENA+, no specific SSc antibodies 9.5% (n=29), >1 SSc specific antibodies 8.9% (n=27).

We evaluated distribution of clinical features and SSc-specific auto-antibodies amongst SSc patients with and without malignancies (Tables 1 and 2). Patients with cancer history were older, had longer duration between first Raynaud phenomenon and first visit to the day-care program, less often diffuse cutaneous disease and more often pulmonary arterial hypertension (Table 1). There were no significant differences in auto-antibody status between patients with or without cancer history, although prevalence of anti-topoisomerase was numerically lower (15.2%, n=7 vs 24.3%, n=63) and RNA polymerase III (10.9%, n=5 vs. 5.0%, n=13) was numerically higher amongst patients with malignancy.

Table 1. Baseline characteristics of patients of the CCISS cohort with recent follow-up, auto-antibody status determined and at least 2 visits to the comprehensive care pathway

	no malignancy n=259	**malignancy n=46**	**p**
male, %(n)	16.2 (42)	15.2 (7)	0.865
age, mean (SD)	53.0 (14.1)	60.9 (13.7)	**0.001**
time since first Raynaud phenomenon, median (IQR)	9.3 (3.8-17.9)	13.6 (5.3-21.0)	**0.024**
time since first non-Raynaud phenomenon, median (IQR)	4.7 (1.6-11.1)	5.3 (2.1-11.7)	0.455
5 year-survival since first non-Raynaud phenomenon, %(n)	79.1(204)	80.4(37)	0.902
unknown, %(n)	17.1 (44)	15.2 (7)	0.767
dcSSc, %(n)	27.0 (70)	13.0 (6)	**0.043**
mRSS, median (IQR)	4.0 (1.3-6.0)	2.0 (0.0-4.5)	0.096
lung involvement on HRCT, %(n)	54.4 (141)	56.5 (26)	0.794
arrhythmia, %(n)	38.2 (95)	47.7 (21)	0.231
PAH, %(n)	2.7 (7)	13.0 (46)	**0.001**
>10% weight loss, %(n)	10.9 (28)	8.7 (4)	0.655
history of renal crisis, %(n)	4.3 (11)	2.2 (1)	0.503
DU, %(n)	23.2 (60)	23.9 (11)	0.912

CCISS cohort: Combined Care in Systemic Sclerosis cohort; Leiden University Medical Center dcSSc - diffuse cutaneous Systemic Sclerosis, DU - digital ulcers, PAH - pulmonary arterial hypertension

Table 2. Auto-antibody prevalences of patients with and without cancer history of the CCISS cohort with recent follow-up, auto-antibody status determined and at least 2 visits to the comprehensive care pathway

	no malignancy n=259	**malignancy n=46**	**p**
anti-topoisomerase, %(n)	27.4 (71)	17.4 (8)	0.153
anti-centromere, %(n)	37.8 (98)	39.1 (18)	0.868
anti-RNA polymerase III, %(n)	5.8 (15)	10.9 (5)	0.200
anti-U1RNP, %(n)	9.3 (24)	8.7 (4)	0.902767
anti-U3RNP, %(n)	4.6 (12)	2.2 (1)	0.447
anti-Pm/Scl, %(n)	6.9 (18)	6.5 (3)	0.916
anti-ThTo, %(n)	1.2 (3)	4.3 (2)	0.116
anti-Ku, % (n)	1.9 (5)	2.2 (1)	0.913
>1 disease specific auto-antibody, %(n)	8.9 (23)	8.7 (4)	0.968
ANA/ENA negative, %(n)	4.6 (12)	2.2 (1)	0.447
ANA/ENA positive, no SSc specific auto-antibody, %(n)	8.9 (23)	13.0 (6)	0.375

Unfortunately, we could not replicate the finding of Bernal-Bello et al. of Pm/Scl being more prevalent in SSc patients with a diagnosis of cancer. For Pm/Scl frequencies were similar between patients with (6.5%, n=3) and without malignancy (6.9%, n=18, p=0.916).

Also, our research is limited in its design as it concerns a single center cohort with limited sample size, especially for auto-antibodies with lower prevalences in general. However, based on our data we cannot advocate comprehensive cancer screening for Pm/Scl positive SSC patients.

In addition, interestingly, the authors hypothesize that, as has been shown for RNApIII antibodies (3), changed expression of the antigen targeted by Pm/Scl in cancer tissue, might trigger the auto-immune response, and result in Pm/Scl positive systemic sclerosis as a paraneoplastic phenomenon. However, a consequence of auto-antibodies directed against proteins that are highly or differently expressed in tumor tissue might also be relevant in preventing tumor progression and metastasis. In cancer, changed expression of proteins known as antigens in SSc, is described not only for RNApIII and Pm/Scl, but is also described for anti-topoisomerase (ATA) and anti-centromere (ACA) (4-6).

Indeed, the incidence of cancer in SSc is known to be increased compared to the general population (7, 8). We therefore hypothesize that prevalence of auto-antibodies differ between SSc patients with and without cancer diagnosis according to their potency to fight cancer in a preclinical stage.

More research in the association of auto-antibodies and occurrence of cancer amongst SSc patients is warranted, as this may shine light on disease pathogenesis in both diseases. In our opinion, antibody status in its current form cannot help identifying which patients to screen for cancer in the daily clinical setting, therefore clinical manifestations should be leading in which patients to screen.

REFERENCES

1. Bernal-Bello D, de Tena JG, Guillén-del Castillo A, Selva-O'Callaghan A, Callejas-Moraga EL, Marín-Sánchez AM, et al. Novel risk factors related to cancer in scleroderma. Autoimmunity Reviews. 2017.
2. Meijs J, Schouffoer AA, Ajmone Marsan N, Kroft LJ, Stijnen T, Ninaber MK, et al. Therapeutic and diagnostic outcomes of a standardised, comprehensive care pathway for patients with systemic sclerosis. RMD open. 2016;2(1):e000159.
3. Joseph CG, Darrah E, Shah AA, Skora AD, Casciola-Rosen LA, Wigley FM, et al. Association of the autoimmune disease scleroderma with an immunologic response to cancer. Science. 2014;343(6167):152-7.
4. Husain I, Mohler JL, Seigler HF, Besterman JM. Elevation of topoisomerase I messenger RNA, protein, and catalytic activity in human tumors: demonstration of tumor-type specificity and implications for cancer chemotherapy. Cancer Res. 1994;54(2):539-46.
5. Zhang W, Mao J-H, Zhu W, Jain AK, Liu K, Brown JB, et al. Centromere and kinetochore gene misexpression predicts cancer patient survival and response to radiotherapy and chemotherapy. Nature Communications. 2016;7.
6. Marshall L, White RJ. Non-coding RNA production by RNA polymerase III is implicated in cancer. Nature Reviews Cancer. 2008;8(12):911-4.
7. Olesen AB, Svaerke C, Farkas D, Sørensen HT. Systemic sclerosis and the risk of cancer: a nationwide population-based cohort study. Br J Dermatol. 2010;163(4):800-6.
8. Onishi A, Sugiyama D, Kumagai S, Morinobu A. Cancer incidence in systemic sclerosis: meta-analysis of population-based cohort studies. Arthritis Rheum. 2013;65(7):1913-21.

PART III

Anti-topoisomerase I positive systemic sclerosis

5

Prognostic properties of anti-topoisomerase antibodies in patients identified by the ACR/EULAR 2013 Systemic Sclerosis criteria

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Sir,

Auto-antibodies in Systemic Sclerosis (SSc) are important tools for disease prognostication (1). Anti-topoisomerase I antibodies (ATA) are associated with a more severe disease course, diffuse cutaneous involvement (dcSSc) and severe interstitial lung disease (ILD), while anti-centromere antibodies (ACA) are associated with a mild disease course, limited skin involvement (lcSSc) and only rare occurrence of ILD. Hence, one would assume increased mortality in the ATA+ group as compared to the ACA+ group.

Contrasting to these presumed predictive properties, Steen et al. showed that when patients are followed from their first visit to the rheumatologist rather than observed from their first symptom, there is no difference in survival between ATA+ and ACA+ patients (2). This finding might have been the consequence of the lack of sensitivity of the ACR 1980 SSc classification criteria (3) to identify early and limited cutaneous SSc. Indeed, ACA+ patients showed longer symptom duration at diagnosis (mean 7.7 years) compared to ATA+ patients (mean 3.8 years) in the manuscript of Steen et al.

The LeRoy and Medsger 2001 criteria (4) and the ACR/EULAR 2013 criteria (5) enable improved identification of limited cutaneous and early SSc patients. The Leiden Combined Care In Systemic Sclerosis Cohort (CCISS) (6) has, from its beginning, included patients according to these criteria and as such, comprises also early and mild cases not fulfilling the ACR 1980 criteria. Taking advantage of our cohort, we collected longitudinal data of 95 ATA+ and 122 ACA+ patients fulfilling the ACR/EULAR 2013 criteria and compared survival, disease progression and development of severe skin and lung involvement, using Kaplan-Meier and Cox survival analysis. Disease progression was defined as ≥1 of the following: increase in mRSS ≥5 points and ≥25%, worsening of lung involvement with ≥ 10% relative decline in forced vital capacity (FVC) and follow-up FVC <80% of predicted or with ≥ 5% to < 10% relative decline in FVC and a ≥ 15% relative decline in diffusion capacity of the lung (DLCO) with follow-up DLCO <80% of predicted, incident digital ulcers requiring prostacyclin treatment, newly diagnosed myocardial involvement, renal crisis, severe gastro-intestinal symptoms, inflammatory myositis, pulmonary arterial hypertension or mortality. According to the Medsger Disease Severity scale ≥3 (7), severe skin involvement was defined as a modified Rodnan Skin Score (mRSS) ≥30 and severe lung involvement as DLCO or FVC <50% of predicted.

At baseline, ACA+ patients were more often female (n=112/122 [91%] vs. n=68/95 [72 %], p<0.01), older (mean age 58±13 yrs. vs. 52±15 yrs., p <0.01) and numerically had a longer disease duration since their first non-Raynaud symptom (median 3.9 [IQR 1.2-9.9] yrs. vs. 2.8 [IQR 0.8-9.3] yrs., p=0.40). Severe skin involvement was seen in 3 ATA+ and none of the ACA+ patients, severe lung involvement in 23 ATA+ and

17 ACA+ patients. Forty-seven percent of ACA+ patients and 19% of ATA+ patients did not fulfil the ACR 1980 criteria. Longitudinal follow-up was available for 85 ATA+ and 107 ACA+ patients with median follow-up of 4.2 and 3.6 years, respectively. Within this period, 12 ATA+ (14%) and 7 ACA+ patients (7%) died and 44 ATA+ (52%) and 39 ACA+ patients (36%) experienced disease progression. Two ATA+ patients (3%) developed severe skin and 8 (14%) severe lung involvement. Of the ACA+ patients, none developed severe skin or lung involvement; however, 2 patients had lung function deterioration after lobectomy for lung cancer and 5 experienced deterioration without any sign of ILD (on HRCT) or pulmonary arterial hypertension (excluded after right-heart catheterization). Kaplan-Meier curves are presented in Figure 1. Notably, there were no differences in mortality (ATA+ HR 2.0 95%CI 0.7-5.2, ref ACA+) and disease progression (ATA+ HR 1.3 95%CI 0.8-2.1, ref ACA+) after correction for age at baseline, sex and time since first non-Raynaud. Differences in the development of severe skin and lung progression could not be assessed by Cox regression, as they did not occur in the ACA+ subset.

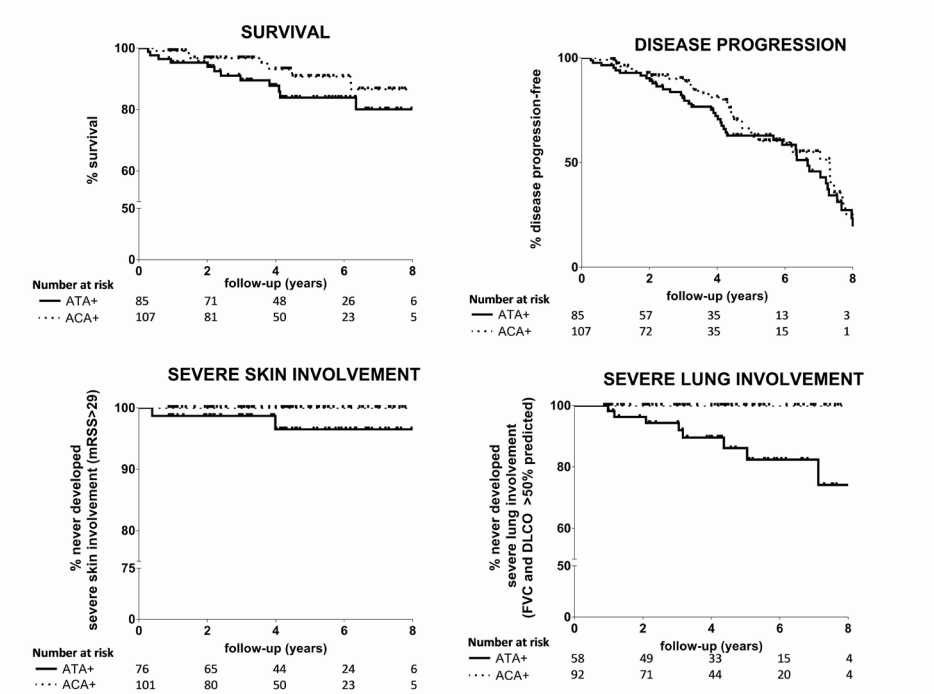

Figure 1. Comparison of survival, disease progression development of severe skin and development of severe lung involvement between ACA+ and ATA+ patients over time

Our data indicate that the introduction of the ACR/EULAR 2013 criteria has not resulted in improved prognostic properties of ACA and ATA in terms of mortality or disease progression. Still, ACA+ and ATA+ patients are phenotypically distinct. Hence, it is likely that the ACR/EULAR 2013 criteria lead to the identification of additional ATA+ patients with less severe disease. This notion is supported by the observation that mortality in the ACA+ subset is comparable to the findings reported by Steen et al. and because only 4 of the 17 ATA+ patients that were additionally identified received aggressive immunosuppression (either mycophenolate mofetil, cyclophosphamide or hematopoietic stem cell transplantation) during follow-up.

In conclusion, our findings suggest that ATA+ patients additionally identified using the ACR/EULAR 2013 criteria are not solely those identified earlier, but also include patients with a less severe disease course. Consequently, additional biomarkers are needed in SSc to guide clinical practice and patient selection for clinical trials.

REFERENCES

1. Nihtyanova SI, Denton CP. Autoantibodies as predictive tools in systemic sclerosis. Nat Rev Rheumatol. 2010;6(2):112-6.
2. Steen VD, Powell DL, Medsger TA, Jr. Clinical correlations and prognosis based on serum autoantibodies in patients with systemic sclerosis. Arthritis Rheum. 1988;31(2):196-203.
3. Association AR. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Arthritis Rheum. 1980;23(5):581-90.
4. LeRoy EC, Medsger TA, Jr. Criteria for the classification of early systemic sclerosis. J Rheumatol. 2001;28(7):1573-6.
5. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/ European League against Rheumatism collaborative initiative. Arthritis Rheum. 2013;65(11):2737-47.
6. Meijs J, Schouffoer AA, Ajmone Marsan N, Kroft LJ, Stijnen T, Ninaber MK, et al. Therapeutic and diagnostic outcomes of a standardised, comprehensive care pathway for patients with systemic sclerosis. RMD open. 2016;2(1):e000159.
7. Medsger TA, Jr., Bombardieri S, Czirjak L, Scorza R, Della Rossa A, Bencivelli W. Assessment of disease severity and prognosis. Clin Exp Rheumatol. 2003;21(3 Suppl 29):S42-6.

6

The effect of sex on outcomes in systemic sclerosis: does anti-topoisomerase I status matter? A EUSTAR analysis

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Abstract

Background
Male Systemic Sclerosis (SSc) patients more often express anti-topoisomerase antibodies (ATA) compared to female patients. We present an in-depth analysis on the effects of sex on SSc outcomes, independent of autoantibody status.

Methods
Using Kaplan Meier curves and Cox proportional hazard models, we evaluated the independent effect of sex on mortality and on the incidence of diffuse skin involvement (dcSSc), interstitial lung disease (ILD) and pulmonary hypertension (PH) in SSc in two cohorts: 1. the Leiden Combined Care In SSc cohort (CCISS; n=242) and 2. the EUropean Scleroderma Trial and Research cohort (EUSTAR; n=4263). We profited from the large sample size of the EUSTAR cohort to perform multivariate analyses including adjustment for autoantibody, age and race and accounting for left-truncation.

Results
SSc males more often express ATA than SSc females (CCISS: 40% vs 21%; EUSTAR: 49% vs 38%). EUSTAR based analyses showed that male sex was associated with mortality (HR 2.6 [95% CI 2.0-3.4]) and its effect was stronger than the effect of ATA (HR 1.33 [95% CI 1.0-1.8]). Male sex was also independently associated with development of dcSSc (HR 1.4 [95%CI 1.1-1.8]) and PH (HR 1.5 [95%CI 1.2-2.0]). Only for ILD the effect of ATA (HR 1.9 [95%CI 1.5-2.5]) was stronger than the effect of sex (HR 1.1 [95%CI 0.9-1.3]).

Conclusions
Male sex is strongly associated with mortality in SSc. This association cannot be explained by a higher prevalence of ATA among males.

Introduction

Systemic sclerosis (SSc) is a rare and heterogeneous disease, clinically characterized by Raynaud's phenomenon, skin and pulmonary fibrosis and cardiac and gastro-intestinal dysfunction (1). The disease is characterized by a complex pathophysiology (2). Dysregulation of the immune system is evidenced by the presence of specific antinuclear antibodies that have clinical and prognostic associations (2, 3). For example: anti-topoisomerase I antibodies (ATA) are associated with diffuse cutaneous involvement and occurrence of interstitial lung disease (ILD) (3-6), while anti-centromere antibodies (ACA) are associated with limited cutaneous involvement, gastrointestinal involvement and a lower likelihood of significant ILD (6-8).

While females are overrepresented in SSc (female: male approximately 5 :1), male sex is associated with early and increased mortality and with presence of ILD (5, 9). Interestingly, the prevalence of SSc-specific autoantibodies also differs with sex: In the EUSTAR cohort, prevalence of ACA is 31% among females and 10% among males, while prevalence of ATA is 31% among females and 54% among males (9-11). Based on this sex-specific distribution of SSc specific auto-antibodies, it could be hypothesized that at least part of the differences observed between male and female patients with SSc are explained by differences in autoantibody distribution.

Our aim was to evaluate the effect of sex on mortality and development of diffuse cutaneous skin involvement (dcSSc), ILD and PH, not explained by autoantibody status. To this end, we took advantage of two cohorts: The Leiden Combined Care In Systemic Sclerosis cohort (CCISS) and the EULAR Scleroderma Trials and Research (EUSTAR) prospective multicenter systemic sclerosis cohort. Using Kaplan Meier curves, with stratification of patients into 6 risk-groups according to sex and autoantibody status (i. ACA+ female, ii. ACA+ male, iii. ATA-ACA- female, iv. ATA-ACA-male, v. ATA+ female and vi. ATA+ male) and Cox regression analysis, this study gains insight in the risks of sex and autoantibodies independently. The analyses performed here are unique in the field, as we adjusted for left-truncation to correct for various disease durations at cohort entrance.

Methods

Leiden Combined Care in Systemic Sclerosis
Data from consecutive SSc patients included in the Combined Care in Systemic Sclerosis Cohort (CCISS) of the Leiden University Medical Center, Leiden, The Netherlands between April 1st, 2009 and June 1st, 2016 were analysed. The CCISS cohort comprises annual prospective data collection with local ethics approval, as described previously (12). Unique in this cohort is the standardized and extensive

annual follow-up with high rate of data completeness. Complete results on prevalence of SSc specific autoantibodies including antibodies directed against topoisomerase (ATA) and centromere (ACA) are available in 97% of patients (13).

The EUSTAR cohort
The European Scleroderma Trials and Research group (EUSTAR) database documents a multinational, prospective and dynamic scleroderma cohort with longitudinal follow-up, which started in June 2004. At time of data extraction (March 28th, 2018), data on 14,998 patients were recorded in the database. A detailed description of the cohort is provided elsewhere (4, 14, 15). Participating centers obtained ethics committee approval. The Leiden patients were excluded from the EUSTAR dataset.

Inclusion criteria
From both cohorts, patients meeting the following criteria were included for analysis: 1. fulfilment of the ACR/EULAR 2013 classification criteria for SSc (16), 2. available auto-antibody status (including at least ANA (anti-nuclear antibody), ATA and ACA status), 3. available skin subtyping (as defined by Medsger and Leroy (17), subdivision of patients in limited and diffuse cutaneous SSc), 4. available radiographic assessment of ILD (by either chest X-ray or high resolution computed tomography [HRCT]) at least one time during baseline or follow-up, 5. date of disease onset known (defined as the date of onset of the first non-Raynaud symptom [91% of cases], or when the date of first non-Raynaud symptom was missing, as the date of the first Raynaud symptom [9% of cases]), and 6. no coexisting SSc specific antibodies (ATA, ACA, RNA polymerase III, Pm/Scl, U1RNP, U3RNP). Flowcharts of patient inclusion in both cohorts are shown in Figure 1. Comparing included and excluded patients there were no significant differences.

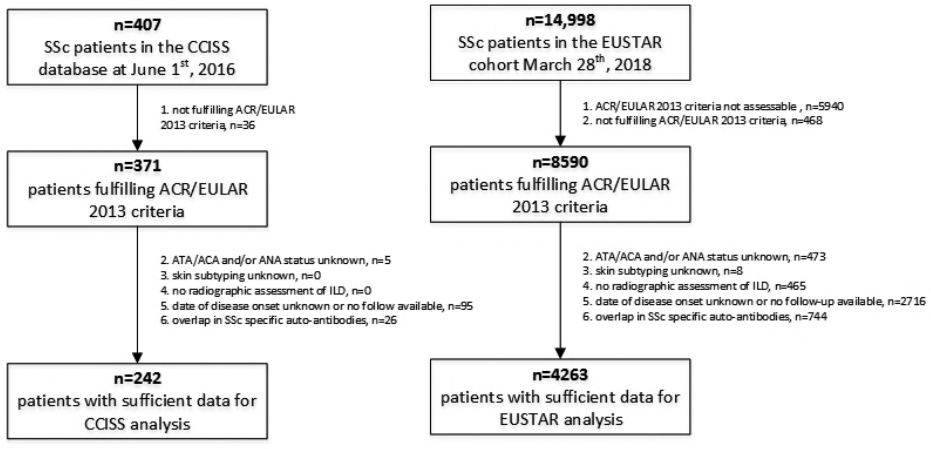

Figure 1. Flowchart of patient inclusion of the Leiden Combined Care in Systemic Sclerosis (CCISS) cohort (left) and EUSTAR cohort (right)

Definitions
Survival time since date of disease onset was registered in each database, including whether death was related to SSc. When a patient once developed dcSSc according to their skin pattern, the patient was classified as dcSSc from that moment onwards, even in case of later improvement to a limited skin pattern. Severe lung involvement was defined as forced vital capacity (FVC) and/or diffusion capacity of the lung for carbon-monoxide (DLCO) of ≤50% of predicted, accompanied by presence of lung fibrosis and/or ground glass opacifications as evaluated by high resolution computed tomography (HRCT). FVC and/or DLCO < 50% was chosen as it corresponds to a score of 2 or higher on the Medsger Disease Severity Scale (18). PH in the CCISS cohort was based on right heart catheterization (RHC); patients were selected for right heart catheterization using the DETECT algorithm (19) and a multidisciplinary team discussion with expert cardiologists, pulmonologists, internal medicine specialists and rheumatologists. In the EUSTAR database PH was registered (yes/no) by the recording physician (based on either echocardiography or RHC).

Statistical analyses
Baseline characteristics of risk-groups (stratified for ATA status, ACA status and sex, i.e., i. ACA+ female, ii. ACA+ male, iii. ATA-ACA- female, iv. ATA-ACA- male, v. ATA+ female and vi. ATA+ male) were compared, testing significancy of differences as appropriate, for both cohorts.

Kaplan Meier methods were used to construct survival curves and in the EUSTAR cohort also for visualization of the development of dcSSc, severe ILD and PH over time. The curves were calculated separately for sex and auto-antibody status and compared using the log rank test. Rates of occurrence of the different outcomes were calculated in different time periods and different risk groups. The Cox proportional hazard model was used to study the effect sex and auto-antibody while adjusting for race and age at disease onset. For all patients, the date of disease onset predated the date of cohort entry (left-truncation). We accounted for this in all analyses to prevent survival bias. Patients were censored at time of last visit or after 10 years of disease duration. The proportional hazards assumption was verified by plotting log minus log survival plots (LML plots) and performance of Schoenfeld's global test. All analyses represent complete case analyses since complete data was an inclusion criterium. Analyses were performed in IBM SPSS Statistics 23. Stata, version 14 (StataCorp LP, College Station, TX USA) was used to account for left-truncation in the survival analyses. Statistical tests were two-sided with an α-level of 0.05.

Table 1. Baseline characteristics and 10-year survival of patients of the Leiden (CCISS) cohort

	overall	females	males	ACA+ female	ACA+ male	ACA-ATA- female	ACA-ATA- male	ATA+ female	ATA+ male
	n=242	**n=190 79%**	**n=52 21%**	**n=83 34%**	**n=10 4%**	**n=67 28%**	**n=21 9%**	**n=40 17%**	**n=21 9%**
median disease duration at cohort entrance (yrs., min-max)	2.4 (0.0-9.8)	2.5 (0.0-9.8)	2.1 (0.0-9.3)	2.5 (0.0-9.8)	1.3 (0.4-8.6)	2.8 (0.1-9.8)	3.0 (0.3-9.2)	1.7 (0.0-9.5)	0.9 (0.0-9.3)
Demographics									
age at disease-onset, mean±SD	51±15	51±15	52±13	53±13	55±14	50±15	49±14	48±17	53±13
caucasian, n (%)	193 (80)	149 (78)	44 (85)	70 (84)	9 (90)	53 (79)	17 (81)	26 (65)	18 (86)
smoking (ever)[1], n (%)	144 (62)	108 (59)	36 (74)	46 (57)	6 (60)	45 (69)	15 (75)	17 (45)	15 (79)
disease features									
dcSSc, n (%)	61 (25)	37 (19)	24 (46)	2 (2)	0 (0)	21 (31)	8 (38)	14 (35)	16 (76)
severe ILD, n (%)	17 (7)	25 (13)	17 (33)	3 (4)	1 (10)	15 (22)	9 (43)	7 (18)	7 (33)
PH, n (%)	17 (7)	12 (6)	5 (10)	8 (10)	0 (0)	4 (6)	2 (10)	0 (0)	3 (14)
renal crisis, n (%)	8 (3)	6 (3)	2 (4)	0 (0)	0 (0)	5 (7)	1 (5)	1 (3)	1 (5)
10-year survival, n (%)	220 (91)	178 (94)	42 (81)	81 (98)	8 (80)	61 (99)	18 (86)	36 (90)	16 (76)

ACA=anti-centromere antibodies; ATA=anti-topoisomerase I antibodies; CCISS=combined care in Systemic Sclerosis; dcSSc=diffuse cutaneous Systemic Sclerosis; ILD=interstitial lung disease; max=maximum; min=minimum; PH=pulmonary hypertension; SD= standard deviation; yrs=years
1 missing in n=9

Results

1. The Leiden Combined Care in Systemic Sclerosis (CCISS) cohort

Of 242 CCISS patients included (Figure 1), 52 were male and 190 were female. This patient population comprised 83 ACA+ females (34%), 10 ACA+ males (4%), 67 ATA-ACA- females (28%), 21 ATA-ACA- males (9%), 40 ATA+ females (17%) and 21 ATA+ males (9%). Baseline characteristics are presented in Table 1. The autoantibody distribution differed significantly between men and women: expression of ATA occurred significantly more often in males compared to females (40 vs. 21%, p<0.01) while ACA expression was significantly more common in females (44 vs. 19% p<0.01). At cohort entry severe ILD (33 vs. 13%, p=0.01) and dcSSc (46 vs. 19%, p=0.01) were more frequent in males compared to females.
During 800 person-years of follow-up (125 for males, 583 for females), 22 patients died (10 males, 12 females). Mortality in males was higher than in females (log-rank p<0.01; data presented in Table 1). After stratification for sex, no significant differences in survival between the three autoantibody groups were observed (log rank in male subgroups analyses p=0.53; female subgroup analyses p=0.16).

2. The EUSTAR cohort

2.1 Male and female distribution of auto-antibodies
To further replicate and deepen the data described above, we next performed a similar analysis in the independent EUSTAR cohort. A total of 4263 patients from the EUSTAR database were included (Figure 1). The included patient set comprised 1380 ACA+ females (32%), 130 ACA+ males (3%), 777

Table 2. Baseline characteristics of EUSTAR Systemic Sclerosis patients

	overall	**male**	**female**	**ACA+ female**	**ACA+ male**	**ACA-ATA- female**	**ACA-ATA- male**	**ATA+ female**	**ATA+ male**
	n=4263	**n=783 18%**	**n=3480 82%**	**n=1380 32%**	**n=130 3%**	**n=777 18%**	**n=272 6%**	**n=1323 31%**	**n=381 9%**
disease duration									
median disease duration at cohort entry (min-max)	3.0 (0.0-10.0)	2.1 (0.0-9.9)	3.2 (0.0-10.0)	3.5 (0.0-10.0)	3.2 (0.0-9.8)	2.7 (0.0-9.9)	1.9 (0.0-9.9)	3.2 (0.0-10.0)	2.0 (0.0-9.9)
demographics									
age at disease-onset, mean±SD	48.8±14.0	49.3±13.3	48.8±14.1	52.5±12.9	53.6±12.8	47.2±14.1	50.3±13.5	45.8±14.4	47.2±13.0
race									
white, n (%)	3592 (84)	699 (89)	2893 (83)	1202 (87)	123 (95)	625 (80)	242 (89)	1066 (81)	334 (88)
black, n (%)	54 (1)	5 (1)	49 (1)	8 (1)	0 (0)	25 (3)	3 (1)	49 (4)	2 (1)
asian, n (%)	94 (2)	9 (1)	85 (2)	15 (1)	0 (0)	21 (3)	3 (1)	16 (1)	6 (2)
other/ undefined, n (%)	523 (12)	70 (9)	453 (13)	155 (11)	7 (5)	106 (14)	24 (9)	192 (15)	39 (10)
smoking (ever), n (%)[a]	1210 (36)	388 (63)	822 (30)	361 (31)	72 (63)	257 (41)	144 (66)	204 (20)	172 (60)
disease-specific features									
dcSSc, n (%)	1638 (39)	422 (55)	1216 (35)	101 (7)	14 (11)	349 (45)	147 (55)	766 (58)	261 (69)

Table 2. Baseline characteristics of EUSTAR Systemic Sclerosis patients *(continued)*

	overall	**male**	**female**	**ACA+ female**	**ACA+ male**	**ACA-ATA- female**	**ACA-ATA- male**	**ATA+ female**	**ATA+ male**
	n=4263	**n=783 18%**	**n=3480 82%**	**n=1380 32%**	**n=130 3%**	**n=777 18%**	**n=272 6%**	**n=1323 31%**	**n=381 9%**
severe ILD[b], n (%)	496 (21)	146 (31)	350 (19)	41 (10)	11 (29)	88 (20)	40 (25)	221 (21)	95 (34)
pulmonary hypertension, n (%)[c]	538 (14)	108 (16)	430 (14)	189 (15)	16 (15)	82 (12)	34 (15)	159 (13)	58 (17)
renal crisis, n (%)	76 (2)	21 (3)	55 (2)	12 (1)	0 (0)	23 (3)	17 (6)	20 (2)	4 (1)

ACA=anti-centromere autoantibody, ATA=anti-topoisomerase I autoantibody; dcSSc=diffuse cutaneous Systemic Sclerosis; ILD=interstitial lung disease

[a] missing in 866 (20%); [b] missing in 1899 (45%) [c] missing in 131 (3%); [d] missing in 39 (1%)

ATA- ACA- females (18%), 272 ATA- ACA- males (6%), 1323 ATA+ females (31%), and 381 ATA+ males (9%). Baseline characteristics are presented in Table 2. Males were more often ATA positive compared to females (49% vs. 38%, p<0.01), and females were more frequently ACA positive compared to males (40% vs. 17%, p<0.01), confirming the findings in the CCISS cohort.

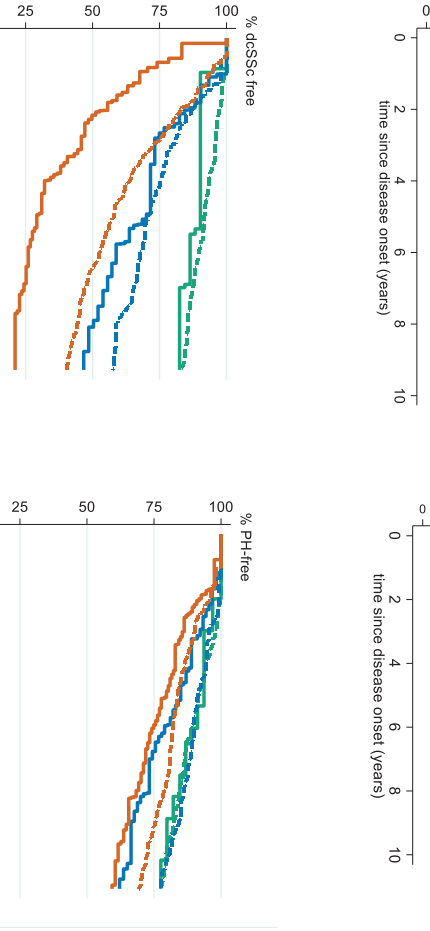

Fig 2. Kaplan Meier curves for survival, development of severe ILD and diffuse cutaneous involvement according to gender and autoantibodies

2.1 Mortality, diffuse cutaneous skin involvement, interstitial lung disease and pulmonary hypertension according to sex and autoantibody derived risk-groups

An overview of available data and events in the EUSTAR cohort is shown in Tables 3. During 15953 person-years of follow-up (2795 for males and 13158 for females) 263 patients died (100 males, 163 females). Kaplan Meier survival curves (Figure 2) show

Table 3. Number of patients developing the outcome of interest and patient years observed in the EUSTAR cohort

	overall	**ACA+ female**	**ACA+ male**	**ACA-ATA- female**	**ACA-ATA- male**	**ATA+ female**	**ATA+ Male**
	n=4263	n=1380	n=130	n=777	n=272	n=1323	n=381
10-year survival analysis							
patient years observed	15953	5382	435	2840	999	4936	1360
deaths, $n/n_{at\ risk}$ (%)	263/4263 (6)	47/1380 (3)	8/130 (6)	34/777 (4)	38/272 (14)	82/1323 (6)	54/381 (14)
development of severe ILD analysis*							
severe ILD at cohort entrance, n (%)[a]	496 (21)	41 (10)	11 (29)	88 (20)	40 (25)	221 (21)	95 (34)
patient years observed	10809	4254	356	1906	688	2870	735
development of severe ILD, $n/n_{at\ risk}$ (%)	337/3064 (11)	40/1137 (4)	5/104 (5)	58/548 (11)	25/192 (13)	161/855 (19)	48/228 (21)
development of dcSSc analysis*							
dcSSc at cohort entrance, n (%)[b]	1638 (39)	101 (7)	14 (11)	349 (45)	147 (55)	766 (59)	261 (69)
patient years observed	8663	4666	367	1324	346	1674	286

Table 3. Number of patients developing the outcome of interest and patient years observed in the EUSTAR cohort *(continued)*

	overall	**ACA+ female**	**ACA+ male**	**ACA-ATA- female**	**ACA-ATA- male**	**ATA+ female**	**ATA+ Male**
	n=4263	n=1380	n=130	n=777	n=272	n=1323	n=381
development of dcSSc, $n/n_{at\ risk}$ (%)	399/2524 (16)	92/1244 (7)	6/108 (6)	75/413 (18)	28/114 (25)	156/528 (30)	42/117 (36)
development of PH analysis							
PH at cohort entrance, n (%)[c]	538 (14)	189 (15)	16 (15)	82 (12)	34 (15)	159 (13)	58 (17)
patient years observed	11251	3888	318	2093	643	3443	866
development of PH, $n/n_{at\ risk}$ (%)	391/3081 (13)	114/1032 (11)	9/87 (10)	57/568 (10)	33/177 (19)	130/962 (14)	48/255 (19)

ACA=anti-centromere autoantibody, ATA=anti-topoisomerase I autoantibody; dcSSc=diffuse cutaneous Systemic Sclerosis; ILD=interstitial lung disease

* $n_{at\ risk}$ and n at cohort entrance do not sum up to n=4263 because of patients lacking baseline or follow-up assessments on skin or pulmonary involvement and therefore exclusion in analysis

[a] missing in 1899 (45%); [b]missing in 39 (1%); [c] missing in 446 (10%)

that survival in males is worse, at all time points within all auto-antibody groups. The same trend was observed for SSc related mortality (Supplementary Material).
The risk to develop dcSSc was highest among ATA+ males, followed by ATA+ females. DcSSc was rare in ACA+ patients, both males and females. Development of ILD was highest in ATA+ males, followed by ATA+ females. Development of pulmonary hypertension is seen most often in ATA-ACA- males and ATA+ subjects, in which males developed PH more often than females (log-rank p=0.03).

2.2 Independent association of sex with SSc outcomes

To evaluate the independent effect of sex on survival and disease outcomes, multivariate left-truncated Cox regression analyses with correction for age, race and gender were performed (Table 4). Interaction between sex and autoantibody status was not statistically significant for any of the outcomes. Both sex and ATA positivity were associated with mortality (male HR 2.6; ATA+ HR 1.3), dcSSc (male HR 1.4; ATA+ HR 1.7) and PH (male HR 1.5; ATA+ HR 1.4) after adjustment for age and race.
Multivariate cox regression analysis confirmed that development of severe ILD is associated with ATA+ (HR 1.9, 95%CI 1.5-2.5), but not with sex (male sex HR 1.1, 95%CI 0.9-1.3).

Table 4. Hazard ratios for mortality, development of severe interstitial lung disease and development of diffuse cutaneous involvement and pulmonary hypertension

	univariate unadjusted HR (95% CI)	p	multivariate model (95%CI)	p
A. MORTALITY				
male	2.9 (2.3-3.8)	<0.01	2.6 (2.0-3.4)	<0.01
ACA-	2.2 (1.6-2.9)	<0.01	2.0 (1.4-2.9)	<0.01
ATA+	1.7 (1.3-2.1)	<0.01	1.3 (<1.0-1.8)	0.06
age at onset (per 10 yrs. increase of age)	1.7 (1.5-1.8)	<0.01	1.8 (1.6-2.0)	<0.01
race (ref=caucasian)				
asian	0.7 (0.2-2.3)	0.59	1.1 (0.3-3.3)	0.92
black	0.7 (0.2-3.0)	0.67	1.4 (0.4-5.8)	0.62
other/undefined	0.6 (0.4-0.9)	0.02	0.7 (0.5-1.1)	0.13
B. SEVERE INTERSTITIAL LUNG DISEASE				
male	1.5 (1.1-1.9)	<0.01	1.1 (0.9-1.3)	0.25
ACA-	4.7 (3.4-6.4)	<0.01	3.3 (2.3-4.8)	<0.01
ATA+	3.2 (2.5-4.0)	<0.01	1.9 (1.5-2.5)	<0.01

Table 4. Hazard ratios for mortality, development of severe interstitial lung disease and development of diffuse cutaneous involvement and pulmonary hypertension *(continued)*

	univariate unadjusted HR (95% CI)	p	multivariate model (95%CI)	p
age at onset (per 10 yrs. increase of age)	1.1 (<1.0-1.2)	0.11	1.2 (1.1-1.3)	<0.01
race (ref=caucasian)				
asian	0.9 (0.3-2.4)	0.8	1.1 (0.4-2.9)	0.88
black	2.4 (1.1-5.4)	0.03	2.4 (1.1-5.6)	0.03
other/undefined	1.4 (1.1-1.8)	0.04	1.3 (0.9-1.7)	0.14
C. DIFFUSE CUTANEOUS INVOLVEMENT				
male	1.7 (1.4-2.2)	<0.01	1.4 (1.1-1.8)	0.01
ACA-	4.2 (3.3-5.2)	<0.01	2.8 (2.1-3.8)	<0.01
ATA+	3.3 (2.7-4.0)	<0.01	1.7 (1.3-2.1)	<0.01
age at onset (per 10 yrs. increase of age)	0.9 (0.8-0.9)	<0.01	1.0 (0.9-1.0)	0.21
race (ref=caucasian)				
asian	1.9 (0.9-3.9)	0.07	2.3 (1.2-4.7)	0.02
black	3.6 (1.5-8.7)	<0.01	3.2 (1.3-7.7)	0.01
other/undefined	1.3 (1.0-1.8)	0.04	1.3 (0.9-1.7)	0.12
D. PULMONARY HYPERTENSION				
male	1.6 (1.2-2.0)	<0.01	1.5 (1.2-2.0)	0.01
ACA-	1.3 (1.1-1.6)	0.01	1.2 (0.9-1.6)	0.13
ATA+	1.4 (1.1-1.6)	<0.01	1.4 (1.1-1.8)	0.01
age at onset (per 10 yrs. increase of age)	1.4 (1.4-1.6)	<0.01	1.5 (1.4-1.7)	<0.01
race (ref=caucasian)				
asian	0.6 (0.2-1.8)	0.34	0.8 (0.2-2.4)	0.65
black	0.8 (0.2-2.4)	0.65	1.3 (0.4-3.9)	0.70
other/undefined	1.0 (0.7-1.3)	0.82	1.1 (0.8-1.5)	0.54

ACA=anti-centromere antibody; ATA=anti-topoisomerase I antibody; HR=hazard ratio
Interactions ('male*ATA+' and 'male*ACA-') were checked, but non-significant.

Discussion

Our data confirms, in two different SSc cohorts, that autoantibody distribution differs with sex, with males being more often ATA+ and less often ACA+ compared to females. Although the population of SSc mainly consists of females, males show increased mortality. In our analyses, we demonstrate that increased mortality among males cannot be explained by a different auto-antibody distribution, being more often ATA+. Notably, the survival of ATA+ females is better than the survival of any of the male risk-groups. Specifically, the presented multivariate analyses show that male sex is the factor with the strongest effect on survival. Additionally, we show that also dcSSc and PH occur more often in SSc males compared to females, independent of autoantibodies. On the contrary, development of ILD is most strongly associated with ATA positivity.

Strikingly, although males comprise only 19% of the total population under study, males account for 39% of all deaths. Currently, the factors underlying this observed morbidity-mortality paradox are not clear. We can only speculate that sex hormones and male-female differences in microcirculation, immunity actors, environmental factors and/or fibroblasts may be involved (20, 21). A morbidity-mortality sex paradox has been observed in several diseases that share features observed in SSc, such as idiopathic PAH (iPAH)(22), interstitial pulmonary fibrosis (IPF) (23) and systemic lupus erythematosus (SLE) (24). In iPAH, hemodynamics are worse in male patients, with higher right arterial pressures and lower cardiac index observed (22). Possibly, more increased endothelial stiffness is present in male SSc. This might affect the lethality of complications such as PAH and ILD in SSc, but may also lead to increased cardiovascular events not directly related to SSc in male subjects. As these sex differences also occur in the bleomycin mouse model for SSc (21), further research in this laboratory setting may help to elucidate the underlying factors explaining more severe disease in males.

In line with our observations, various other studies have indicated that males with SSc have a worse prognosis than females with SSc (4, 5, 25, 26). We confirm the results of Wangkaew et al. (27) and Hoffman-Vold et al. (7), showing that male sex is not influencing the development of ILD, taking into account auto-antibody status. Like our study, a previous EUSTAR analysis evaluating mortality in SSc, also showed a gender gap in SSc survival and PH occurrence (26). However, the investigators hypothesized that the gender gap might reflect increased comorbidity in males, as in their analyses, SSc related mortality was comparable between males and females. The latter might be due to analyses approach taken as the evaluation of SSc-related mortality only included patients that died during follow-up. In addition, the analysis did not account for left-truncation, lead-time and survival bias.

Survival in SSc seems to improve when the time to diagnosis shortens (28). However, we and other authors observed that SSc males have a shorter time to diagnosis than female SSc patients (29, 30). This, therefore, is likely not explaining the sex paradox observed. Nevertheless, this information is important for the interpretation of other studies that identify predictors of mortality: As male patients tend to be diagnosed earlier and die from complications early in the disease course (indicating a higher prevalence of rapidly progressive disease), the chances for males to be included in inception cohorts are increased compared to prevalent cohorts. In incident cohorts, mild SSc cases may be underrepresented, as a delay between first symptoms and confirmation of diagnosis is more likely to occur, while for inclusion the duration of non-Raynaud's may not exceed the defined time period for incident disease. Influenced by this bias, studies identifying predictors of mortality and progressive disease in inception cohorts recognize male sex as a risk factor for mortality (5, 31), while similar studies in prevalent cohorts (not taking into account survival and lead time bias) do not (32, 33). The analysis we present in this study, with survival analysis using adjustment for left-truncation to correct for possible survival bias is therefore additive to the field, providing the opportunity to approach the effects of sex and auto-antibody status on disease outcomes in a more balanced way.

Our analyses have also limitations, which should be taken into account. For the current study, we did not consider other autoantibodies than ATA and ACA. We chose to focus on ACA antibodies and ATA antibodies as these are most prevalent and cover 75% of the population under study. We did not address male-female differences that might be present in other auto-antibody groups (such as RNA polymerase III and Pm/Scl). Presence of other, yet unknown or unmeasured, auto-antibodies in these risk groups cannot be ruled out, but given the rarity of co-expression of different auto-antibodies in SSc it is unlikely to influence the results. Also, in the identification of PH, it is likely some of the identified cases in fact represent false positives, as in EUSTAR PH is defined as a yes/no variable based on echocardiographic findings instead of right heart catheterization. Moreover, although we selected patients fulfilling the ACR/EULAR 2013 classification criteria, aiming to include also milder cases, selection bias might still have occurred by a possible lower inclusion of very mild SSc in the EUSTAR cohort; 50% of the EUSTAR population had diffuse skin involvement. Finally, based on predefined inclusion criteria, we had to exclude 50% of the existing EUSTAR cohort. However, as we specifically chose to focus on the independent effects of sex and antibody status, we preferred a complete case analysis. At the same time, the current study demonstrates the importance and possibilities offered by the EUSTAR database enabling complex survival analyses in a rare and heterogeneous disease. Moreover, in the CCISS cohort the same observations were made, while exclusion in this cohort was mainly based on short follow-up instead of missing data.

To conclude, male sex is an independent and strong risk factor for mortality in SSc and additionally is independently associated with diffuse skin fibrosis and PH. This indicates that sex-factors contribute to the disease phenotype and the lethality of disease complications. These observations therefore point to the possibility to influence sex-related factors for therapy.

Supporting information

Supplementary data can be obtained by contacting the first author.

References

1. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. N Engl J Med. 2009;360(19):1989-2003.
2. Kuwana M, Kaburaki J, Okano Y, Tojo T, Homma M. Clinical and prognostic associations based on serum antinuclear antibodies in Japanese patients with systemic sclerosis. Arthritis Rheum. 1994;37(1):75-83.
3. Nihtyanova SI, Denton CP. Autoantibodies as predictive tools in systemic sclerosis. Nat Rev Rheumatol. 2010;6(2):112-6.
4. Walker U, Tyndall A, Czirjak L, Denton C, Farge-Bancel D, Kowal-Bielecka O, et al. Clinical risk assessment of organ manifestations in systemic sclerosis: a report from the EULAR Scleroderma Trials And Research group database. Ann Rheum Dis. 2007;66(6):754-63.
5. Hao Y, Hudson M, Baron M, Carreira P, Stevens W, Rabusa C, et al. Early Mortality in a Multinational Systemic Sclerosis Inception Cohort. Arthritis & rheumatology. 2017;69(5):1067-77.
6. Steen VD. Autoantibodies in systemic sclerosis. Semin Arthritis Rheum. 2005;35(1):35-42.
7. Hoffmann-Vold AM, Aalokken TM, Lund MB, Garen T, Midtvedt O, Brunborg C, et al. Predictive value of serial high-resolution computed tomography analyses and concurrent lung function tests in systemic sclerosis. Arthritis & rheumatology. 2015;67(8):2205-12.
8. Hoffmann-Vold AM, Molberg O, Midtvedt O, Garen T, Gran JT. Survival and causes of death in an unselected and complete cohort of Norwegian patients with systemic sclerosis. J Rheumatol. 2013;40(7):1127-33.
9. Peoples C, Medsger TA, Jr., Lucas M, Rosario BL, Feghali-Bostwick CA. Gender differences in systemic sclerosis: relationship to clinical features, serologic status and outcomes. J Scleroderma Relat Disord. 2016;1(2):177-240.
10. Carreira PE, Carmona L, Joven BE, Loza E, Andreu JL, Riemekasten G, et al. Gender differences in early systemic sclerosis patients: a report from the EULAR scleroderma trials and research group (EUSTAR) database. Clin Exp Rheumatol. 2018;36 Suppl 113(4):68-75.
11. Freire M, Rivera A, Sopena B, Tolosa Vilella C, Guillen-Del Castillo A, Colunga Arguelles D, et al. Clinical and epidemiological differences between men and women with systemic sclerosis: a study in a Spanish systemic sclerosis cohort and literature review. Clin Exp Rheumatol. 2017;35 Suppl 106(4):89-97.
12. Meijs J, Schouffoer AA, Ajmone Marsan N, Kroft LJ, Stijnen T, Ninaber MK, et al. Therapeutic and diagnostic outcomes of a standardised, comprehensive care pathway for patients with systemic sclerosis. RMD open. 2016;2(1):e000159.
13. Markusse IM, J.; de Boer, B.; Bakker, J.; Schippers, P.; Schouffoer, A.; Ajmone Marsan, N.; Kroft, L.; Ninaber, M.; Huizinga, T.; de Vries-Bouwstra, J. The Additive Value of Nailfold Videocapillaroscopy Patterns to Disease-Specific Autoantibodies in Discrimination of Patients with Systemic Sclerosis at Risk for Severe Organ Involvement. Ann Rheum Dis. 2015;74(Supp 2):597.
14. Tyndall A, Ladner UM, Matucci-Cerinic M. The EULAR Scleroderma Trials and Research Group (EUSTAR): an international framework for accelerating scleroderma research. Curr Opin Rheumatol. 2008;20(6):703-6.
15. Tyndall A, Mueller-Ladner U, Matucci-Cerinic M. Systemic sclerosis in Europe: first report from the EULAR Scleroderma Trials And Research (EUSTAR) group database. Ann Rheum Dis. 2005;64(7):1107.
16. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. Arthritis Rheum. 2013;65(11):2737-47.

17. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Jr., et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol. 1988;15(2):202-5.
18. Medsger TA, Jr., Bombardieri S, Czirjak L, Scorza R, Della Rossa A, Bencivelli W. Assessment of disease severity and prognosis. Clin Exp Rheumatol. 2003;21(3 Suppl 29):S42-6.
19. Coghlan JG, Denton CP, Grünig E, Bonderman D, Distler O, Khanna D, et al. Evidence-based detection of pulmonary arterial hypertension in systemic sclerosis: the DETECT study. Ann Rheum Dis. 2014;73(7):1340-9.
20. Ciaffi J, van Leeuwen N, Schoones J, Huizinga T, de Vries-Bouwstra J, editors. Sex hormones and sex hormone-targeting therapies in systemic sclerosis: a systematic literature review. Semin Arthritis Rheum; 2019: Elsevier.
21. Ruzehaji N, Avouac J, Elhai M, Frechet M, Frantz C, Ruiz B, et al. Combined effect of genetic background and gender in a mouse model of bleomycin-induced skin fibrosis. Arthritis Res Ther. 2015;17:145.
22. Ventetuolo CE, Praestgaard A, Palevsky HI, Klinger JR, Halpern SD, Kawut SM. Sex and haemodynamics in pulmonary arterial hypertension. Eur Respir J. 2014;43(2):523-30.
23. Gribbin J, Hubbard RB, Le Jeune I, Smith CJ, West J, Tata LJ. Incidence and mortality of idiopathic pulmonary fibrosis and sarcoidosis in the UK. Thorax. 2006;61(11):980-5.
24. Crosslin KL, Wiginton KL. Sex differences in disease severity among patients with systemic lupus erythematosus. Gend Med. 2011;8(6):365-71.
25. Hussein H, Lee P, Chau C, Johnson SR. The effect of male sex on survival in systemic sclerosis. The Journal of rheumatology. 2014;41(11):2193-200.
26. Elhai M, Avouac J, Walker UA, Matucci-Cerinic M, Riemekasten G, Airo P, et al. A gender gap in primary and secondary heart dysfunctions in systemic sclerosis: a EUSTAR prospective study. Ann Rheum Dis. 2016;75(1):163-9.
27. Wangkaew S, Euathrongchit J, Wattanawittawas P, Kasitanon N, Louthrenoo W. Incidence and predictors of interstitial lung disease (ILD) in Thai patients with early systemic sclerosis: Inception cohort study. Mod Rheumatol. 2016;26(4):588-93.
28. Nihtyanova SI, Tang EC, Coghlan JG, Wells AU, Black CM, Denton CP. Improved survival in systemic sclerosis is associated with better ascertainment of internal organ disease: a retrospective cohort study. QJM. 2010;103(2):109-15.
29. Delisle VC, Hudson M, Baron M, Thombs BD, And The Canadian Scleroderma Research Group A. Sex and time to diagnosis in systemic sclerosis: an updated analysis of 1,129 patients from the Canadian scleroderma research group registry. Clin Exp Rheumatol. 2014;32(6 Suppl 86):S-10-4.
30. Hudson M, Thombs B, Baron M. Time to diagnosis in systemic sclerosis: is sex a factor? Arthritis Rheum. 2009;61(2):274-8.
31. Domsic RT, Nihtyanova SI, Wisniewski SR, Fine MJ, Lucas M, Kwoh CK, et al. Derivation and External Validation of a Prediction Rule for Five-Year Mortality in Patients With Early Diffuse Cutaneous Systemic Sclerosis. Arthritis & rheumatology. 2016;68(4):993-1003.
32. Meijs J, Schouffoer AA, Marsan NA, Stijnen T, Putter H, Ninaber MK, et al. A prediction model for progressive disease in systemic sclerosis. RMD open. 2015;1(1):e000113.
33. Avouac J, Walker UA, Hachulla E, Riemekasten G, Cuomo G, Carreira PE, et al. Joint and tendon involvement predict disease progression in systemic sclerosis: a EUSTAR prospective study. Ann Rheum Dis. 2016;75(1):103-9.

7

Association of anti-topoisomerase I antibodies of the IgM Isotype with disease progression in anti-topoisomerase I-positive Systemic Sclerosis

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Abstract

Background
Anti-topoisomerase I auto-antibodies (ATA) in systemic sclerosis (SSc) are associated with diffuse skin involvement and interstitial lung fibrosis. Thus far, however, the relations between the ATA response and disease course have not yet been fully evaluated.

Objectives
To gain insight into the relation between characteristics of the ATA immune response and clinical disease course in ATA+ SSc.

Methods
ATA-IgG, -IgM and -IgA levels were assessed in consecutive serum samples of baseline ATA-IgG+ patients from the Leiden Combined Care In Systemic Sclerosis cohort (CCISS). One-year disease progression was defined by a relevant increase in modified Rodnan Skin Score (mRSS), decline in pulmonary function tests, development of digital ulcers, renal crisis, pulmonary hypertension and/or mortality. Validation was performed in ATA+ SSc patients from the Oslo University Hospital and University Hospital Zurich.

Results
Of 103 ATA-IgG+ patients available in the CCISS cohort, 81 patients had clinical data available to assess one-year disease progression. Of these 81 patients, 23 patients (28%) showed disease progression. At baseline, disease-progressors were significantly more often ATA-IgM+ compared to non-progressors (21/23 [91%] vs 33/58 [57%], p<0.01). This finding was confirmed in the independent validation samples.

Conclusion
In ATA-IgG+ SSc patients, presence of ATA-IgM, which might be taken as a surrogate for an ongoing auto-reactive B cell immune response, is associated with disease progression.

Introduction

Anti-topoisomerase I antibodies (ATA) are highly specific for Systemic Sclerosis (SSc) (1). Patients with isolated Raynaud's phenomenon have an increased risk of progression to SSc when ATA positive (2), indicating presence of ATA in a preclinical phase. In established SSc, ATA are associated with diffuse cutaneous SSc (dcSSc), severe interstitial lung disease (ILD) and their presence indicates an unfavorable prognosis (3-7). This association with a typical clinical phenotype suggests that the immune response involved in ATA production may play a role in disease pathophysiology. The exact pathogenicity of ATA, however, has not yet been elucidated.

In daily clinical practice, ATA+ SSc is heterogeneous. Not all ATA+ patients demonstrate a severe disease course, some patients experience only moderate skin and lung fibrosis (6, 8). Based on the hypothesis that topoisomerase I represents a candidate autoantigen in the pathogenesis of SSc, different groups have studied immunization with topoisomerase I in mouse models. These models demonstrated that a specific antibody response can be induced, resulting in varying extents of fibrosis in skin and lungs of immunized mice (9, 10).

Previous small studies in SSc have shown that IgG and IgA levels of ATA correlate with skin scores (11-13). Loss of the ATA response, on the other hand, has been associated with a favorable disease course in a small patient group (14). However, the relations between ATA isotype profile and isotype levels and disease course have not yet been fully evaluated in larger SSc cohorts. By taking advantage of our well described SSc cohort with annual, prospective and comprehensive clinical data available, we investigated the association between presence and levels of ATA-IgG, -IgA and -IgM and disease course in ATA-IgG+ SSc.

Patients and Methods

Patient population
The Combined Care in Systemic Sclerosis (CCISS) cohort Leiden is a prospective cohort that started in April 2009, including all consecutive SSc patients evaluated at the Leiden University Medical Center(15). Ethical approval for data collection was obtained from the local ethics committee (CME number B16.037). All participants provided written informed consent. This research was done without patient involvement. As described previously (15), all patients undergo annual extensive screening during a 1 to 2 day health care program, including detailed physical examination, modified Rodnan skin score (mRSS) assessment (16), laboratory testing (with autoantibody screening at baseline), pulmonary function test and optionally:

echocardiography (mandatory at baseline), holter evaluation (mandatory at baseline), cardiopulmonary exercise tests (CPET) and high-resolution computed tomography (HRCT) (mandatory at baseline). Patients are requested to fill in the Scleroderma Health Assessment Questionnaire (HAQ) (17), Short Form-36 (SF-36) (18, 19), Mouth Handicap in Systemic Sclerosis scale (MHISS) (20, 21), EuroIQol-5D (EQ-5D) (22, 23) and Scleroderma Clinical Trial Consortium Gastrointestinal Tract Instrument 2.0 (SCTC GIT 2.0) (24, 25) questionnaires at every visit. Additionally, every visit serum samples are collected and stored in the Leiden Scleroderma Biobank. All patients entering the cohort before September 24th, 2016, who were ATA-IgG+ were selected for the present study. Only patients who had a clinical SSc diagnosis at inclusion and fulfilled the ACR/EULAR 2013 SSc classification criteria (26) at any point during their disease course were evaluated.

Disease progression

Clinical data were collected, with censoring at January 1st, 2018. Skin progression was defined as a ≥5 point and ≥25% increase in mRSS (27). Worsening of lung involvement was defined as ≥ 10% relative decline in forced vital capacity (FVC) with follow-up FVC <80% predicted or ≥ 5% to < 10% relative decline in FVC and either a ≥ 15% relative decline in DLCO with follow-up DLCO <80% predicted or increase of lung involvement as determined by HRCT, towards >20% lung involvement (28). Patients were considered disease progressors in case of skin- and/or lung progression, incident digital ulcers (DU), newly diagnosed myocardial involvement, scleroderma renal crisis, pulmonary hypertension (PH) or in case of death. Use of aggressive immunosuppression in both progressors and non-progressors was assessed, including hematopoietic stem cell transplantation (HSCT), cyclophosphamide and mycophenolate mofetil.

Anti-topoisomerase I assay and measurements

Total ATA-IgG, -IgA, and -IgM levels of consecutive samples collected before January 1st, 2017 were measured in baseline and follow-up sera by fluorescence enzyme-linked immune sorbent assay [FEIA], using Phadia250® system [Thermo Fisher Scientific, Nieuwegein, The Netherlands]. If necessary, sera were diluted to obtain a reliable ATA isotype-specific level. For ATA-IgG, the manufacturer specified a cut-off value of 7 aU/mL. For ATA-IgA and ATA-IgM, no manufacturer cut-off values were available. Therefore, sera of 51 non-rheumatic subjects were measured and the cut-off value was determined as the mean plus two standard deviations of the measurements. A cut-off for ATA-IgM was determined at 432 aU/mL and for ATA-IgA at 77 aU/mL. To evaluate specificity of the assay, ATA-isotype levels from 5 ANA+ SSc patients lacking SSc specific antibodies and from 5 ACA+ SSc patients were additionally assessed. None of these patients were positive for any of the isotypes in the ATA assay.

Data validation

For validation of the main findings, baseline serum samples of ATA-IgG+ patients from the Oslo University Hospital (29) and from the University Hospital Zurich (30) were tested for the presence and levels of ATA isotypes using the same methodology. Baseline and follow-up clinical data were additionally collected. At both centers longitudinal data of SSc patients is being collected according to the EUSTAR recommendations (31). Details of these cohorts can be found elsewhere (29, 30). Collection and analysis of biomaterial and their clinical associations have been approved by the Cantonal Ethics Committee in Switzerland (PB_2016-02014 and BASEC-Nr. 2018-01873) and by the Data Protection Authority in Norway (No.2006/119). All patients provided informed consent.

Statistical analysis

Descriptive statistics were used to characterize the study population clinically. Contingency tables were evaluated by Fisher's exact, c^2 or Mann-Whitney test as appropriate. Correlations between isotype levels were assessed by Spearman's correlation coefficient. Disease progression over time was analysed by Kaplan-Meier survival analysis. P values <0.05 were considered significant. To exclude relevant bias by expression of ATA-IgM and ATA-IgA in patients negative for ATA-IgG and by evaluating a higher cut-off for ATA-IgM to define positivity, a sensitivity analysis was performed (Supplementary Figure 5). In addition, we evaluated the robustness of the data by re-analyze using a different cut off for ATA IgM. Statistical analysis was performed using SPSS version 23.0 and GraphPad Prism 7.

Results

Baseline characteristics and ATA isotype expression of the population under study

In total, 103 ATA-IgG+ patients from the CCISS cohort were included. Of these patients, a total of 333 samples were available (range 1-8 per patient). Sixty-nine percent of patients were female with a mean age of 53 years, and 48% had diffuse cutaneous SSc (Table 1). At baseline, median duration since first non-Raynaud's symptom was 2.8 years. Clinical follow-up was available for 3.4 years (range 0.0-8.4).All but one patient evaluated were ATA-IgA+ at baseline. This patient was also low in his ATA-IgG level (24 aU/mL). At baseline, 65% (n=67/103) of patients were ATA-IgM+. Antibody isotype levels at baseline correlated weakly (ATA-IgG and ATA-IgM [r_s=0.25, p=0.01], ATA-IgG and ATA-IgA [r_s=0.30, p=<0.01], ATA-IgA and ATA-IgM [r_s=0.45, p=<0.01]) and were not correlated with disease duration (Supplementary Figure 1 + 2). Correlations between baseline ATA isotype levels and skin scores are presented in Figure 1, levels of ATA-IgG correlated with skin scores (r_s=0.41, p<0.01), other isotypes did not correlate with skin scores.

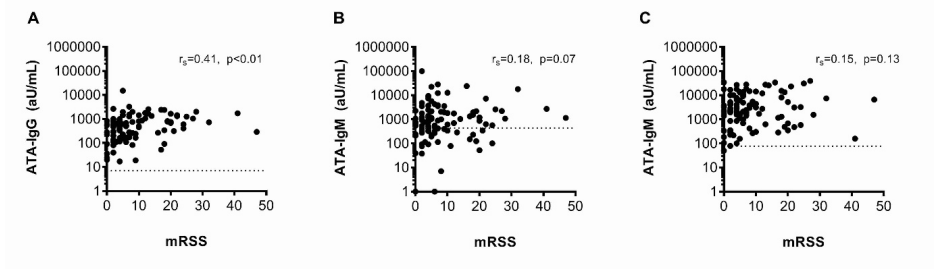

Figure 1. Correlations between baseline levels of ATA-IgG (panel A), ATA-IgM (panel B) and ATA-IgA (panel C) and modified Rodnan Skin Score (mRSS) of patients from the Leiden Combined Care in Systemic Sclerosis (CCISS) cohort (n=103). ATA-IgG levels correlate with skin scores (rs=0.41, p<0.01). [ATA=anti-topoisomerase I].

During follow-up, 12 patients died (combined pulmonary and cardiac failure n=6, cardiac ischemia n=1, sepsis during hematopoietic stem cell transplant work-up n=1, gastro-intestinal ischemia n=1, influenza-related n=1, multi-organ failure during acute myeloid leukemia treatment n=1, unclear n=1).

Loss and gain of ATA-isotype response is only frequent for ATA-IgM

Change of isotype profile over time was assessed in 75 patients as 28 patients did not have follow-up samples available (Table 2, Supplementary Figure 3). Of these patients, four patients showed a loss of the ATA-IgG+ response (5%); all four were ATA-IgM- at baseline. Two of these patients were treated with IV cyclophosphamide before baseline sampling, one was treated with HSCT before baseline sampling and one was treated with HSCT 3 months after baseline sampling. Three of these four patients were ATA-IgA+ at baseline and two of them also showed loss of the ATA-IgA response. In total, there were 4 patients that lost ATA-IgA response over time (5%), of whom one also lost the ATA-IgM response, but remained ATA-IgG+. Loss and gain ATA-IgM response over time was more common compared to other isotypes. Thirty-one percent (n=14/45) of patients lost and 10% (n=3/29) of ATA-IgM- patients gained an ATA-IgM+ response when followed from baseline.

Table 1. Baseline characteristics of all anti-topoisomerase I-IgG+ Systemic Sclerosis patients in the study

	all patients (n=103)
Demographic	
female, n(%)	70 (68)
age, mean[yrs.]±SD	53.0±14.8
smoking (ever), n(%)	50 (49)
Disease duration	
since onset first Raynaud symptom, median [yrs.] (IQR)	5.8 (2.1-13.4)
since onset first non-Raynaud symptom, median [yrs.] (IQR)	2.8 (0.8-9.3)
Organ involvement	
dcSSc, n(%)	49 (48)
modified Rodnan Skin Score, median (IQR)	6 (2-12)
FVC, mean [% of predicted] ±SD	87±27
DLCO, mean [% of predicted] ±SD	63±17
history of renal crisis, n(%)	3 (3)
digital ulcers, n(%)	14 (14)
pulmonary hypertension, n(%)	5 (5)
Previous use of immunosuppression*	
HSCT, n(%)	7 (7)
CYC (ever), n(%)	24 (23)
MMF (ever), n(%)	1 (1)

ATA=anti-topoisomerase antibodies, CYC=cyclophosphamide, dcSSc=diffuse cutaneous Systemic Sclerosis, DLCO=diffusing capacity of the lung, FVC=forced vital capacity, HSCT=hematopoietic stem cell transplantation, IQR=interquartile range, MMF=mycophenolate mofetil, SD=standard deviation, yrs.=years

* Immunosuppression= use of either HSCT, CYC or MMF

Table 2. Changes in presence of anti-topoisomerase I isotypes in paired (first and last available serum) samples of 75 ATA-IgG SSc patients with follow-up samples available

	ATA isotype status at baseline/last follow-up			
	+/+* (n)	+/-* (n)	-/-* (n)	-/+* (n)
ATA-IgG	71	4	-	-
ATA-IgM	31	14	27	3
ATA-IgA	70	4	1	0

ATA=anti-topoisomerase I autoantibody
*Status of the first available serum sample/status of the last available serum sample

Disease progression is more frequent in ATA-IgG+ SSc patients positive for ATA-IgM

To assess the association between ATA isotype profile and disease progression, we used data of 81 patients with one-year clinical follow-up available. During the first year starting from sampling, none of these patients received HSCT, 16 patients were treated with cyclophosphamide and 7 received mycophenolate mofetil.

In total 23 patients showed disease progression according to predefined criteria. This consisted of death (n=4; including combined pulmonary and cardiac failure n=3 and , multi-organ failure during acute myeloid leukemia treatment n=1), skin progression (n=12), lung progression (n=4), digital ulcers (n=5). None of the patients developed clinically meaningful myocardial involvement or renal crisis. Correlations between ATA isotype levels at baseline and one-year change in mRSS, FVC and DLCO % predicted are shown in Supplementary Figure 4. Baseline levels of ATA-IgM and ATA-IgA correlated with a decrease in FVC % predicted, and ATA-IgM additionally correlated with a decrease in DLCO % predicted. Baseline levels of ATA-IgG, -IgM and -IgA were not correlated with one-year change in mRSS.

Table 3. Baseline characteristics of ATA-IgG+ patients stratified according to one-year disease progression

	progressors (n=23)	**non-progressors** (n=58)	**p**
Demographic			
female, n(%)	14 (61)	39 (67)	0.59
age, mean[yrs.]±SD	55.3±16.3	51.9±13.9	0.21
smoking (ever), n(%)	12 (52)	30 (52)	0.95

Table 3. Baseline characteristics of ATA-IgG+ patients stratified according to one-year disease progression *(continued)*

	progressors (n=23)	**non-progressors** (n=58)	**p**
Disease duration			
since onset first Raynaud symptom, median [yrs.] (IQR)	3.8 (1.3-8.4)	5.6 (2.1-12.9)	0.21
since onset first non-Raynaud symptom, median [yrs.] (IQR)	1.9 (0.6-4.5)	3.5 (0.7-11.4)	0.07
Organ involvement			
dcSSc, n(%)	12 (52)	28 (48)	1.00
modified Rodnan Skin Score, median (IQR)	6 (2-19)	6 (3-13)	0.86
FVC, mean [% of predicted] ±SD	89±26	89±28	0.92
DLCO, mean [% of predicted] ±SD	62±18	64±16	0.83
history of renal crisis, n(%)	0 (0)	2 (4)	1.00
digital ulcers, n(%)	0 (0)	5 (9)	0.31
pulmonary hypertension, n(%)	2 (9)	2 (4)	0.59
Previous use of immunosuppression*			
HSCT, n(%)	0 (0)	7 (12)	0.18
CYC (ever), n(%)	4 (17)	16 (28)	0.34
MMF (ever), n(%)	1 (4)	0 (0)	0.28
Use of aggressive immunosuppression* during one-year follow-up			
HSCT, n(%)	0 (0)	0 (0)	-
CYC, n(%)	11 (19)	5 (26)	0.52
MMF, n(%)	1 (5)	6 (10)	0.67
ATA characteristics			
IgG level [aU/mL], median(IQR)	813 (542-1263)	396 (115-832)	<0.01
IgA positivity, n(%)	23 (100)	57 (98)	1.00
IgA level [aU/mL], median(IQR)	9898 (2743-16656)	2045 (462-5314)	<0.01
IgM positivity, n(%)	21 (91)	33 (57)	0.04
IgM level [aU/mL], median(IQR)	1065 (869-3853)	588 (223-1610)	0.01

ATA=anti-topoisomerase antibodies, CYC=cyclophosphamide, dcSSc=diffuse cutaneous Systemic Sclerosis, DLCO=diffusing capacity of the lung, FVC=forced vital capacity, HSCT=hematopoietic stem cell transplantation, IQR=interquartile range, MMF=mycophenolate mofetil, SD=standard deviation, yrs.=years
* Aggressive immunosuppression= use of either HSCT, CYC or MMF.
In 22 patients, clinical follow-up data was not available; therefore, they could not be stratified into either progressors or non-progressors.

In total, 23 patients (28%) showed disease progression according to pre-specified criteria during the first year. Clinical characteristics and ATA isotype profiles at baseline stratified for disease progression are presented in Table 3. At baseline, there were no differences in clinical characteristics between patients with and those without disease progression. Treatment strategy was also comparable between patients with and without disease progression. Strikingly, while the clinical characteristics were similar, ATA isotype levels at baseline were significantly higher and ATA-IgM positivity was significantly more frequent in patients with disease progression (91% vs 57%, p<0.01). Kaplan-Meier analysis underlined the prognostic value of ATA-IgM positivity (Log-Rank - Mantel-Cox p=0.02, Figure 2). Sensitivity analysis did not influence these results (Supplementary Figure 5).

Validation in other cohorts

To confirm our results, we additionally performed ATA isotype level measurements in 90 ATA-IgG+ SSc patients (n=60 from University Hospital Zurich and n=30 from Oslo University Hospital). Baseline characteristics of these patients are presented in Supplementary Table 1. Cross-sectional analysis confirmed the correlation between ATA-IgG levels and skin scores at baseline (r_s=0.37 p=<0.01). Additionally, in this sample set a correlation between ATA-IgG and respectively FVC (r_s=-0.30 p=<0.01) and DLCO (r_s=-0.24 p=0.03) was found. Clinical follow-up at one year was available in 63 patients of the validation samples. During this year, 5 patients died, skin progression was observed in 6, lung progression in 7, incident renal crisis developed in 1 and digital ulcers developed in 5 patients. In total, 24 patients from the validation sample set experienced disease progression. Again, there were no clinical differences between disease progressors and non-progressors at baseline, but disease progressors more often expressed ATA-IgM (96%, vs. 71%, p=0.04) (Supplementary Table 1). Thus, these data confirm that ATA-IgG+ SSc patients that are also positive for ATA-IgM, have a higher risk for disease progression as compared to ATA-IgG+ patients not positive for ATA-IgM.

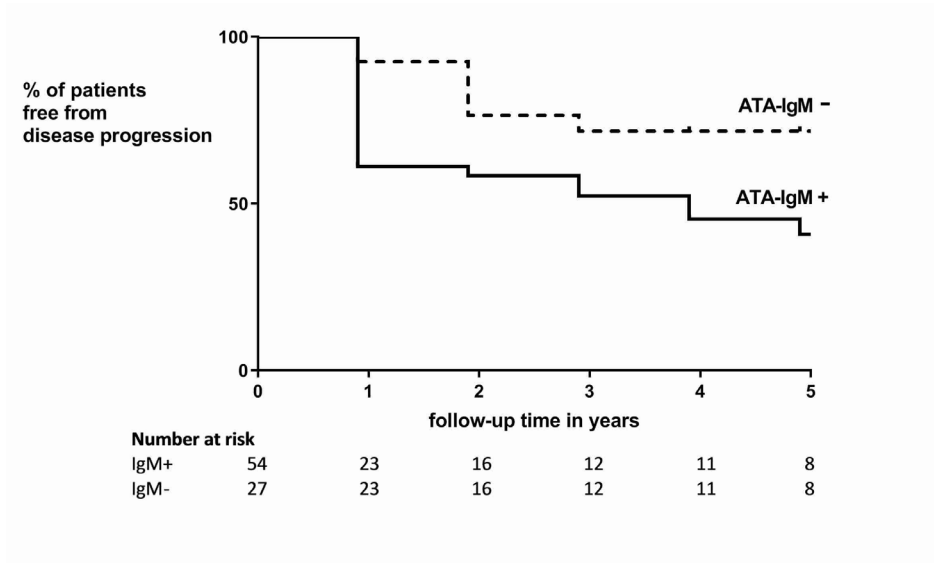

Figure 2. Disease progression over in time in ATA-IgM positive and negative SSc patients from the Leiden Combined Care in Systemic Sclerosis (CCISS) cohort with at least one year follow-up available (n=81). Disease progression occurs more often in ATA-IgM positive patients (Log-Rank - Mantel-Cox p=0.02). [ATA=anti-topoisomerase I].

Discussion

This study shows that ATA-IgG+ SSc patients, additionally positive for ATA-IgM, more often experience disease progression compared to ATA-IgG+, ATA-IgM- patients. Importantly, progressors could not be identified based on baseline clinical parameters. In addition, we show that ATA-IgG+ patients are almost always ATA-IgA+. Alteration from a positive to negative response (or vice versa) for ATA-IgG and ATA-IgA isotypes is relatively rare, while loss and gain of the ATA-IgM response occurs frequently. Over one-third of patients ATA-IgM+ at baseline, becomes ATA-IgM- during follow up.

Our observations of high levels of ATA-IgA, and only part of ATA-IgG+ patients harboring ATA-IgM+, are consistent with previous findings from the early 1990's (32, 33). The sustained ATA-IgG response found in SSc patients, with little or no fluctuations with disease activity and not sero-reverting (in some cases even after high dose cyclophosphamide treatment in the context of hematopoietic stem cell transplantation), points to the notion that this response is long-lived and that its generation depends on T cell help. Hence, it is conceivable that long-lived plasma cells secreting ATA-IgG without the need for antigenic triggering may be responsible for a large fraction of the ATA-IgG levels measured in serum. However, we consider

it possible that there is also a short-lived, more dynamic part of the ATA-response, triggered due to the continuous presence of autoantigens and potentially, additional/external (yet unknown) triggers such as TLR-ligands. Such triggers would be able to recruit naïve B cells from the repertoire and explain why IgM-secreting plasma cells arise that, due to their short life span (i.e., the lack of a long-lived memory compartment) and the short half-life of IgM, more closely reflect disease-relevant processes, with possible clinical consequences in the near future.

ATA-IgG levels have previously been described to be correlated with skin scores (11-13). A study of Kuwana et al. in 28 SSc patients reported that 21% of ATA-IgG+ patients lost their ATA-IgG response over time, which was associated with a favorable disease course (14). Notably, although non-significant, none of these patients were ATA-IgM+ at baseline, while one-third of patients that persisted to be ATA-IgG also were ATA-IgM+ at baseline. In our cohort, loss of ATA-IgG response over time was less common (5%). This discrepancy between the study of Kuwana et al. and ours might be explained by methodological differences. Kuwana et al. used a cut-off of 3 times the standard deviation of samples of healthy controls for their ELISA assays (34). We used a cut-off for ATA-IgG as pre-specified by the manufacturer and used in clinical routine, which corresponds to the mean plus 8 standard deviations (data not shown). Consequently, Kuwana et al. might have included patients with already lower ATA-IgG levels at baseline. In addition, in another study including 21 patients, decreasing levels were accompanied by skin showing atrophic changes, while increasing levels were associated with new onset or worsening of organ involvement. Thus, our work and that from others show that the ATA-response is related to disease course. Nonetheless, the frequency of ATA-IgM+ in patients not experiencing disease progression implicates that ATA-IgM status solely is not sufficient to function as a biomarker in every day clinical practice, but might be of additional help for clinical trial enrichment. As disease progression is highly unlikely in patients negative for ATA-IgM (<10%), ATA-IgM status might be of help to decide to refrain from aggressive treatment like HSCT.

Our hypothesis that ATA or its underlying immune response is (at least partly) responsible for clinical heterogeneity, might not seem to rhyme with the heterogeneity observed among patients who are all ATA-IgG+ and ATA-IgM+ from the first measurement onwards. A pathophysiologic explanation of the clinical heterogeneity within ATA-IgG+, ATA-IgM+ SSc might be found in the presence of additional triggers for ATA or its underlying immune response to become pathogenic. For example, it has been speculated that ATA triggers adhesion and activation of monocytes by binding to DNA-topoisomerase I expressed on fibroblasts. This potentially could lead to amplification of the fibrogenetic cascade (35-37). In line with this, it is tempting to speculate that the presence of ATA may only be pathogenic in case there is insufficient clearance of apoptotic bodies of endothelial cells containing DNA-topoisomerase I. Consequently, the production of ATA-IgG might be an ongoing process in all

ATA+ patients, however if not accompanied by the presence of extracellular DNA-topoisomerase I, the ability of ATA to trigger fibrosis is lost. Clinically, this might result in different ATA+ subsets of patients depending on the level of endothelial cell apoptosis. This could also fit with the observation that more severe capillary loss is associated with more severe organ involvement independent of auto-antibody subtype (38). Alternatively, other characteristics of ATA or its underlying immune response, such as epitope recognition patterns, the extent of T-cell and/or B-cell activation or interaction with cytokines could be important for pathogenicity.

This study has some limitations to be considered. As we only included patients positive for ATA-IgG at baseline, we cannot exclude that there might be patients positive for ATA-IgM and/or ATA-IgA solely. However, based on our sensitivity analysis, we conclude that in SSc patients continuous expression of ATA-IgM without switching to ATA-IgG does hardly occur. Also, as data were derived from a cohort study, treatment was uncontrolled. However, significant treatment differences between disease progressors and non-progressors were not observed. In addition, because of the exploratory character of the study, we deliberately did not correct for multiple testing as this would lead to increased chances of false negative findings (39), which cannot be easily justified in an explorative study. Instead, we validated our main findings in an independent cohort. Finally, we used a composite of several individually validated scores for different organs to define overall disease progression, including all-cause mortality. We acknowledge that a precise determination of cause of death is often difficult, leading to weak data quality. To address this, recorded causes of death are described in the results section. Using a composite end point for disease progression is common in SSc studies (40, 41), as the heterogeneous nature of the disease with multiple organs involved implicates the use of composite indices. Availability of a validated composite for disease progression could have substantiated our findings. However, although it lacks validation, our composite has face validity and most importantly, as our data have been validated in an independent second cohort, our analyses is robust.

In conclusion, our results indicate that the ATA immune response is relevant for the disease course of SSc. Further research of the ATA response by characterization of specific epitopes and other antibody characteristics such as Fc-glycosylation are relevant for understanding of the disease pathogenesis. Most important, our data indicate that expression of an ATA-IgM response associates with an unfavorable disease course, a finding that we validated in other cohorts. Whether IgM positivity of other SSc specific auto-antibodies is of equal importance in explaining disease course remains to be evaluated.

Supporting information

Supplementary data is available at the website of Arthritis & Rheumatology or can be obtained by contacting the first author

REFERENCES

1. Reveille JD, Solomon DH. Evidence-based guidelines for the use of immunologic tests: Anticentromere, Scl-70, and nucleolar antibodies. Arthritis Care Res (Hoboken). 2003;49(3):399-412.
2. Koenig M, Joyal F, Fritzler MJ, Roussin A, Abrahamowicz M, Boire G, et al. Autoantibodies and microvascular damage are independent predictive factors for the progression of Raynaud's phenomenon to systemic sclerosis: a twenty-year prospective study of 586 patients, with validation of proposed criteria for early systemic sclerosis. Arthritis Rheum. 2008;58(12):3902-12.
3. Patterson KA, Roberts-Thomson PJ, Lester S, Tan JA, Hakendorf P, Rischmueller M, et al. Interpretation of an Extended Autoantibody Profile in a Well-Characterized Australian Systemic Sclerosis (Scleroderma) Cohort Using Principal Components Analysis. Arthritis & rheumatology. 2015;67(12):3234-44.
4. Mierau R, Moinzadeh P, Riemekasten G, Melchers I, Meurer M, Reichenberger F, et al. Frequency of disease-associated and other nuclear autoantibodies in patients of the German Network for Systemic Scleroderma: correlation with characteristic clinical features. Arthritis Res Ther. 2011;13(5):R172.
5. Vanthuyne M, Smith V, De Langhe E, Van Praet J, Arat S, Depresseux G, et al. The Belgian Systemic Sclerosis Cohort: correlations between disease severity scores, cutaneous subsets, and autoantibody profile. J Rheumatol. 2012;39(11):2127-33.
6. Steen VD. Autoantibodies in systemic sclerosis. Semin Arthritis Rheum. 2005;35(1):35-42.
7. Khanna D, Denton CP. Evidence-based management of rapidly progressing systemic sclerosis. Best Pract Res Clin Rheumatol. 2010;24(3):387-400.
8. Boonstra M, Ninaber MK, Ajmone Marsan N, Huizinga TWJ, Scherer HU, de Vries-Bouwstra JK. Prognostic properties of anti-topoisomerase antibodies in patients identified by the ACR/EULAR 2013 systemic sclerosis criteria. Rheumatology (Oxford). 2019.
9. Yoshizaki A, Yanaba K, Ogawa A, Asano Y, Kadono T, Sato S. Immunization with DNA topoisomerase I and Freund's complete adjuvant induces skin and lung fibrosis and autoimmunity via interleukin-6 signaling. Arthritis Rheum. 2011;63(11):3575-85.
10. Mehta H, Goulet P-O, Nguyen V, Pérez G, Koenig M, Senécal J-L, et al. Topoisomerase I peptide-loaded dendritic cells induce autoantibody response as well as skin and lung fibrosis. Autoimmunity. 2016;49(8):503-13.
11. Hu PQ, Fertig N, Medsger TA, Jr., Wright TM. Correlation of serum anti-DNA topoisomerase I antibody levels with disease severity and activity in systemic sclerosis. Arthritis Rheum. 2003;48(5):1363-73.
12. Perera A, Fertig N, Lucas M, Rodriguez-Reyna TS, Hu P, Steen VD, et al. Clinical subsets, skin thickness progression rate, and serum antibody levels in systemic sclerosis patients with anti-topoisomerase I antibody. Arthritis Rheum. 2007;56(8):2740-6.
13. Hasegawa M, Imura-Kumada S, Matsushita T, Hamaguchi Y, Fujimoto M, Takehara K. Anti-topoisomerase I antibody levels as serum markers of skin sclerosis in systemic sclerosis. J Dermatol. 2013;40(2):89-93.
14. Kuwana M, Kaburaki J, Mimori T, Kawakami Y, Tojo T. Longitudinal analysis of autoantibody response to topoisomerase I in systemic sclerosis. Arthritis Rheum. 2000;43(5):1074-84.

15. Meijs J, Schouffoer AA, Ajmone Marsan N, Kroft LJ, Stijnen T, Ninaber MK, et al. Therapeutic and diagnostic outcomes of a standardised, comprehensive care pathway for patients with systemic sclerosis. RMD open. 2016;2(1):e000159.
16. Clements P, Lachenbruch P, Siebold J, White B, Weiner S, Martin R, et al. Inter and intraobserver variability of total skin thickness score (modified Rodnan TSS) in systemic sclerosis. J Rheumatol. 1995;22(7):1281-5.
17. Clements PJ, Wong WK, Hurwitz EL, Furst DE, Mayes M, White B, et al. The Disability Index of the Health Assessment Questionnaire is a predictor and correlate of outcome in the high-dose versus low-dose penicillamine in systemic sclerosis trial. Arthritis Rheum. 2001;44(3):653-61.
18. Ware JE, Kosinski M, Keller S. SF-36 physical and mental health summary scales: a user's manual: Health Assessment Lab.; 1994.
19. Newnham EA, Harwood KE, Page AC. Evaluating the clinical significance of responses by psychiatric inpatients to the mental health subscales of the SF-36. J Affect Disord. 2007;98(1):91-7.
20. Mouthon L, Rannou F, Bérezné A, Pagnoux C, Arène J-P, Foïs E, et al. Development and validation of a scale for mouth handicap in systemic sclerosis: the Mouth Handicap in Systemic Sclerosis scale. Ann Rheum Dis. 2007;66(12):1651-5.
21. Schouffoer A, Strijbos E, Schuerwegh M, Mouthon L, Vlieland MV. Translation, cross-cultural adaptation, and validation of the Mouth Handicap in Systemic Sclerosis questionnaire (MHISS) into the Dutch language. Clin Rheumatol. 2013;32(11):1649.
22. Dolan P. Modeling valuations for EuroQol health states. Med Care. 1997;35(11):1095-108.
23. Lamers L, Stalmeier P, McDonnell J, Krabbe P, Van Busschbach J. Kwaliteit van leven meten in economische evaluaties: het Nederlands EQ-5D-tarief. Ned Tijdschr Geneeskd. 2005;149(28):1574-8.
24. Meijs J, Pors D, Vliet VT, Huizinga T, Schouffoer A. Translation, cross-cultural adaptation, and validation of the UCLA Scleroderma Clinical Trial Consortium Gastrointestinal Tract Instrument (SCTC GIT) 2.0 into Dutch. Clin Exp Rheumatol. 2013;32(6 Suppl 86):S-41-8.
25. Khanna D, Hays RD, Park GS, Braun-Moscovici Y, Mayes MD, McNearney TA, et al. Development of a preliminary scleroderma gastrointestinal tract 1.0 quality of life instrument. Arthritis Care Res (Hoboken). 2007;57(7):1280-6.
26. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/ European League against Rheumatism collaborative initiative. Arthritis Rheum. 2013;65(11):2737-47.
27. Khanna D, Furst DE, Clements PJ, Allanore Y, Baron M, Czirjak L, et al. Standardization of the modified Rodnan skin score for use in clinical trials of systemic sclerosis. Journal of Scleroderma and Related Disorders. 2017;2(1):11-8.
28. Khanna D, Mittoo S, Aggarwal R, Proudman SM, Dalbeth N, Matteson EL, et al. Connective Tissue Disease-associated Interstitial Lung Diseases (CTD-ILD) - Report from OMERACT CTD-ILD Working Group. J Rheumatol. 2015;42(11):2168-71.
29. Hoffmann-Vold AM, Midtvedt O, Molberg O, Garen T, Gran JT. Prevalence of systemic sclerosis in south-east Norway. Rheumatology (Oxford). 2012;51(9):1600-5.

30. Frauenfelder T, Winklehner A, Nguyen TD, Dobrota R, Baumueller S, Maurer B, et al. Screening for interstitial lung disease in systemic sclerosis: performance of high-resolution CT with limited number of slices: a prospective study. Ann Rheum Dis. 2014;73(12):2069-73.
31. Jordan S, Maurer B, Toniolo M, Michel B, Distler O. Performance of the new ACR/EULAR classification criteria for systemic sclerosis in clinical practice. Rheumatology (Oxford). 2015;54(8):1454-8.
32. Verheijen R, de Jong BA, van Venrooij WJ. A recombinant topoisomerase I ELISA: screening for IgG, IgM and IgA anti-topo I autoantibodies in human sera. Clin Exp Immunol. 1992;89(3):456-60.
33. Hildebrandt S, Jackh G, Weber S, Peter HH. A long-term longitudinal isotypic study of anti-topoisomerase I autoantibodies. Rheumatol Int. 1993;12(6):231-4.
34. Kuwana M, Medsger TA, Jr., Wright TM. Detection of anti-DNA topoisomerase I antibody by an enzyme-linked immunosorbent assay using overlapping recombinant polypeptides. Clin Immunol Immunopathol. 1995;76(3 Pt 1):266-78.
35. Henault J, Robitaille G, Senecal JL, Raymond Y. DNA topoisomerase I binding to fibroblasts induces monocyte adhesion and activation in the presence of anti-topoisomerase I autoantibodies from systemic sclerosis patients. Arthritis Rheum. 2006;54(3):963-73.
36. Senecal J, Hénault J, Raymond Y. The pathogenic role of autoantibodies to nuclear autoantigens in systemic sclerosis (scleroderma). J Rheumatol. 2005;32(9):1643.
37. Henault J, Tremblay M, Clement I, Raymond Y, Senecal JL. Direct binding of anti-DNA topoisomerase I autoantibodies to the cell surface of fibroblasts in patients with systemic sclerosis. Arthritis Rheum. 2004;50(10):3265-74.
38. Markusse IM, Meijs J, de Boer B, Bakker JA, Schippers HPC, Schouffoer AA, et al. Predicting cardiopulmonary involvement in patients with systemic sclerosis: complementary value of nailfold videocapillaroscopy patterns and disease-specific autoantibodies. Rheumatology (Oxford). 2017;56(7):1081-8.
39. Feise RJ. Do multiple outcome measures require p-value adjustment? BMC Med Res Methodol. 2002;2:8.
40. Sullivan KM, Goldmuntz EA, Keyes-Elstein L, McSweeney PA, Pinckney A, Welch B, et al. Myeloablative Autologous Stem-Cell Transplantation for Severe Scleroderma. N Engl J Med. 2018;378(1):35-47.
41. Avouac J, Walker UA, Hachulla E, Riemekasten G, Cuomo G, Carreira PE, et al. Joint and tendon involvement predict disease progression in systemic sclerosis: a EUSTAR prospective study. Ann Rheum Dis. 2016;75(1):103-9.

PART IV

Systemic Sclerosis: are auto-antibodies our guiding stars?

8

Summary and discussion

In this thesis the role of anti-nuclear auto-antibodies to function as biomarkers in Systemic Sclerosis (SSc) has been evaluated. Respectively, the heterogeneity of the disease, the need for biomarkers and the role for auto-antibodies as such, with specific attention for anti-topoisomerase have been outlined in this thesis.

SSc, is a complex heterogeneous connective-tissue disease that can have a mild disease course, but can also be life-threatening (1). A general introduction on SSc, with discussion of its epidemiologic characters, history, discussion on pathogenesis, diagnosis and classification and treatment is given in Chapter 1. In this chapter also a brief introduction on the Leiden Comprehensive Care Pathway and de Combined Care In Systemic Sclerosis (CCISS) cohort are given, which has been the basis for all research performed within this thesis.

The need for biomarker research

Risk-stratification in SSc is difficult. Exemplary is the disease course of the placebo group in the RITuximab in Systemic sclerosis trial (RITIS-trial) in **Chapter 2.** The trial aimed inclusion of patients at high risk for deterioration, however, was not able to select cases with significant deterioration.

The RITIS trial was a study in which 16 early SSc patients (time of diagnosis <2 years prior to inclusion) were randomized 1:1 to treatment with rituximab (an anti-CD20 B-cell depleting agent) or placebo. As in new-born tight skin mice anti-CD20 treatment development of fibrosis was prevented, while in adult tight-skin mice with already established disease there was no effect of treatment (1), rituximab was hypothesized to terminate the disease process and to have a beneficial effect only in early disease stages. Unfortunately, the trial observed no significant effects. This however does not exclude that some patients may have had a beneficial effect of the treatment with rituximab. Because of the rarity of the disease, performing large scale trials are difficult. In order to study treatment effect in a group as small as possible, selection of patients in which the greatest effects are likely to be observed is required. For a treatment agent that was hypothetically able to stop the disease process and not able to heal, an appropriate selection of patients would imply deterioration in the placebo group. However, as shown from the disease course of the placebo group in the RITIS trial, in which skin scores, lung function and daily functioning were all rather stable, such selection is challenging.

The RITIS trial is not the only trial in SSc that suffers from poor patient selection because of inability to predict patients with progressive disease. For example, the first Scleroderma Lung Study (2) and the Focussced trial (3) showed a relative stable disease course for placebo treated patients. As shown by Table 1 from Chapter 1, SSc-specific auto-antibodies are associated with specific disease features in SSc. Based

on these findings from cross-sectional research, various authors have suggested that auto-antibodies can be used to predict disease course of SSc patients (4-6). The RITIS trial, the SLS I study and also the Focussced did not employ auto-antibodies for patient selection in their inclusion criteria. In our thesis we tried to answer if employment of auto-antibodies for inclusion criteria in such studies should be performed.

Auto-antibodies as biomarkers in Systemic Sclerosis

In **Chapter 3** we evaluated the attributive value of that auto-antibodies in survival prognostication. For this goal we performed a statistical analysis (hierarchical clustering in combination with principal component analysis), in which we let the computer make subgroups of patients based on respectively clinical and demographic characteristics only and subsequently performed the same analysis, only with additional use of auto-antibody status to simulate risk-stratification. Comparing risk-stratification with and without knowledge of auto-antibodies showed that correct prediction of survival within five years increased when the antibody subtypes were included in the model. However, also the number needed to screen increased with 27%, while correct identification of high-risk individuals increased with 13%. This illustrates that although auto-antibodies may associate with survival, its contribution to clinical prognostication when it comes to survival is limited.

Some auto-antibodies in SSc have been described to associate with concurrent malignancies. This is especially the case for RNA polymerase III (RNApIII) (7-9). Therefore, In current disease management, when a patients is newly diagnosed with RNApIII+ SSc, a malignancy screening is performed. For other auto-antibodies their relationship with coincident malignancies is less clear. Bernal-Bello et al. suggested that Pm/Scl antibodies in SSc could also be related to an increased malignancy risk. In **Chapter 4** we show that we could not confirm this finding in the CCISS cohort. Pathophysiologically, the relationship between SSc and cancer might be based on epitope spreading of an immune reaction that was primarily targeted at a transformed oncogene auto-antigen. However, presence of continuous inflammation might also create a situation in which DNA damage more easily emerges with development of cancer as a consequence.

In conclusion, these studies confirm that auto-antibody status only cannot function as an appropriate biomarker in SSc. The urge for a biomarker however is present, not only to select the right patients for clinical trial participation, but also to be able to identify the right patients to monitor more or less closely. Most of these patient will have an anti-topoisomerase I auto-antibody. However as discussed below, within this group further stratification is needed.

Anti-topoisomerase I positive systemic sclerosis

We explored the heterogeneity of ATA positive SSc in **Chapter 5.** We showed that as expected, ATA+ patients in the CCISS cohort more often develop severe pulmonary fibrosis and diffuse skin thickening. Interestingly, when analysed from the time of inclusion in our cohort, in contrast to what one might expect, there was no difference between ATA+ and ACA+ patients in the amount of – and time to disease progression and survival. We were not the first to notice this, also Steen et al. already in 1988 had noticed that when analysed from disease onset, there is a clear difference in survival between ATA+ and ACA+ patients, while survival between ATA+ and ACA+ is similar when assessed from their initial visit to a specialized SSC clinic (10). Although this was recognized in 1988, with the coming of the ACR/EULAR 2013 SSc classification criteria from which is thought to enable diagnosis of patients in an earlier stage (11, 12), we expected that the current clinical practice would be more in line with the analysis from disease onset in 1988. Our analysis revealed that this was however not the case. It seems that the ACR/EULAR 2013 SSc criteria mainly enable diagnosis of mild and not early disease. This became even more clear, by the observation that of all ATA+ patients with longitudinal follow-up ranging up to 8 years, a third of patients never developed fibrotic complications. Additionally, ATA+ patients with normal lung function test at first screening were unlikely to deteriorate to severe lung disease during follow-up. The heterogeneity of ATA+ SSc is as such clearly demonstrated, with a large deal of ATA+ disease under the 2013 criteria being mild.

In SSc, it is remarkable that while the disease is far more prevalent in women, male patients more frequently harbor ATA. We therefore evaluated the prognostic implications of ATA+ and ACA+ separately in men and women in **Chapter 6**. Herein we found that sex is not only associated with the auto-antibody subtype, but is also an independent contributor to disease severity in SSc. Males have increased chances for development of diffuse cutaneous involvement, pulmonary hypertension and disease related mortality. Intensified screening therefore seems adequate in all male SSc patients, independent of auto-antibody status.

In an attempt to recognize when to be alarmed in ATA+ SSc, we investigated whether knowledge of isotypes could be of help to identify patients likely to deteriorate in **Chapter 7.** IgM is an isotype, known to occur in active phases of many diseases. Our finding that ATA-IgM is associated with disease progression, for us therefore confirmed that knowledge of isotype status of specific ANA in SSc might function as additional biomarker. Presence of ATA IgM likely reflects ongoing presentation of disease relevant autoantigens with recruitment of short-lived naïve B cells. But as also part of ATA-IgM+ SSc patients do not deteriorate, there is an ongoing research to factors that lay behind being ATA-IgM+ and that do explain why some patients have stable disease, while others develop these life-threatening complications.

Future perspectives on research in Systemic Sclerosis

Our lack of understanding the disease mechanisms in SSc, hampers the development of successful therapies and cost-effective screening programs. In my opinion, future research therefore should focus on increased understanding of the disease and elucidation of the exact mechanisms that lead to the heterogeneous clinical picture of SSc.

One possibility in to gain better understanding of disease pathophysiology could be the study of patients in clinical remission. As discussed in **Chapter 1** three major contributors in disease pathogenesis of SSc exist: microangiopathy (13, 14), fibrosis (15-18) and immunological changes (19-21). Studying the changes in these three compartments after HSCT might be key to understanding disease mechanisms in SSc.

Another strategy that could provide us with increased understanding of SSc are the clinical trials that are conducted world-wide. Multicenter research, including many patients, do not suffer from insufficient power. Knowledge of the drug-target of the ligand that is tested in a trial with beneficial effect could shine light on disease etiology and equips the treating rheumatologist with strategies in a disease where until know physicians are more or less powerless. For an academic center like the Leiden University Medical Center, being able to participate in trials like the FASST (lanifibranor)(22), and RESOLVE (lenabasum)(23) is therefore priceless.

Biomarkers in Systemic Sclerosis: Are auto-antibodies our guiding stars?

In conclusion, auto-antibody status alone does not provide us with sufficient information to perform risk-stratification in such a way that we can either select the right patients for clinical trials, construct a tailor-made screening program for patients or decide whether and which therapy to start. Still, there are many stumbling blocks ahead in achieving these goals. Nevertheless, we do know that auto-antibodies are clearly associated with the phenotype of the disease. Therefore auto-antibodies might function as one of the guiding stars in SSc follow-up and treatment. However, we are still searching for the total picture in help of navigating. Let's hope, that unlike at the time of Klee (the painter of the work on the cover of this thesis, which represents his work "This star teaches bending" – 1940), in the near future we will no longer have to bend for the star of SSc, but find stars that help us navigate safely through the sometimes calm and peaceful, but possibly also dangerous and unpredictable sea, which the disease course of SSc still is.

REFERENCES

1. Hasegawa M, Hamaguchi Y, Yanaba K, Bouaziz JD, Uchida J, Fujimoto M, et al. B-lymphocyte depletion reduces skin fibrosis and autoimmunity in the tight-skin mouse model for systemic sclerosis. Am J Pathol. 2006;169(3):954-66.
2. Tashkin DP, Elashoff R, Clements PJ, Goldin J, Roth MD, Furst DE, et al. Cyclophosphamide versus placebo in scleroderma lung disease. N Engl J Med. 2006;354(25):2655-66.
3. Khanna D, Lin CJF, Furst DE, Goldin J, Kim G, Kuwana M, et al. Tocilizumab in systemic sclerosis: a randomised, double-blind, placebo-controlled, phase 3 trial. The Lancet Respiratory medicine. 2020;8(10):963-74.
4. Nihtyanova SI, Denton CP. Autoantibodies as predictive tools in systemic sclerosis. Nat Rev Rheumatol. 2010;6(2):112-6.
5. Kuwana M. Circulating Anti-Nuclear Antibodies in Systemic Sclerosis: Utility in Diagnosis and Disease Subsetting. Journal of Nippon Medical School = Nippon Ika Daigaku zasshi. 2017;84(2):56-63.
6. Khanna D, Denton CP. Evidence-based management of rapidly progressing systemic sclerosis. Best Pract Res Clin Rheumatol. 2010;24(3):387-400.
7. Shah AA, Hummers LK, Casciola-Rosen L, Visvanathan K, Rosen A, Wigley FM. Examination of autoantibody status and clinical features associated with cancer risk and cancer-associated scleroderma. Arthritis & rheumatology. 2015;67(4):1053-61.
8. Shah AA, Rosen A, Hummers L, Wigley F, Casciola-Rosen L. Close temporal relationship between onset of cancer and scleroderma in patients with RNA polymerase I/III antibodies. Arthritis Rheum. 2010;62(9):2787-95.
9. Joseph CG, Darrah E, Shah AA, Skora AD, Casciola-Rosen LA, Wigley FM, et al. Association of the autoimmune disease scleroderma with an immunologic response to cancer. Science. 2014;343(6167):152-7.
10. Steen VD, Powell DL, Medsger TA, Jr. Clinical correlations and prognosis based on serum autoantibodies in patients with systemic sclerosis. Arthritis Rheum. 1988;31(2):196-203.
11. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/ European League against Rheumatism collaborative initiative. Arthritis Rheum. 2013;65(11):2737-47.
12. Association AR. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Arthritis Rheum. 1980;23(5):581-90.
13. Koenig M, Joyal F, Fritzler MJ, Roussin A, Abrahamowicz M, Boire G, et al. Autoantibodies and microvascular damage are independent predictive factors for the progression of Raynaud's phenomenon to systemic sclerosis: a twenty-year prospective study of 586 patients, with validation of proposed criteria for early systemic sclerosis. Arthritis Rheum. 2008;58(12):3902-12.
14. Fleischmajer R, Perlish JS. Capillary alterations in scleroderma. J Am Acad Dermatol. 1980;2(2):161-70.
15. Manetti M, Guiducci S, Matucci-Cerinic M. The origin of the myofibroblast in fibroproliferative vasculopathy: does the endothelial cell steer the pathophysiology of systemic sclerosis? Arthritis Rheum. 2011;63(8):2164-7.

16. Kissin EY, Merkel PA, Lafyatis R. Myofibroblasts and hyalinized collagen as markers of skin disease in systemic sclerosis. Arthritis Rheum. 2006;54(11):3655-60.
17. Lafyatis R. Transforming growth factor beta-at the centre of systemic sclerosis. Nature Reviews Rheumatology. 2014;10(12):706-19.
18. Varga J, Abraham D. Systemic sclerosis: a prototypic multisystem fibrotic disorder. J Clin Invest. 2007;117(3):557-67.
19. Tan EM, Rodnan GP, Garcia I, Moroi Y, Fritzler MJ, Peebles C. Diversity of antinuclear antibodies in progressive systemic sclerosis. Anti-centromere antibody and its relationship to CREST syndrome. Arthritis Rheum. 1980;23(6):617-25.
20. Douvas AS, Achten M, Tan EM. Identification of a nuclear protein (Scl-70) as a unique target of human antinuclear antibodies in scleroderma. J Biol Chem. 1979;254(20):10514-22.
21. Sakkas LI, Xu B, Artlett CM, Lu S, Jimenez SA, Platsoucas CD. Oligoclonal T cell expansion in the skin of patients with systemic sclerosis. J Immunol. 2002;168(7):3649-59.
22. Avouac J, Konstantinova I, Guignabert C, Pezet S, Sadoine J, Guilbert T, et al. Pan-PPAR agonist IVA337 is effective in experimental lung fibrosis and pulmonary hypertension. Ann Rheum Dis. 2017:annrheumdis-2016-210821.
23. Spiera R, Hummers L, Chung L, Frech T, Domsic R, Hsu V, et al. OP0006 Safety and efficacy of lenabasum (JBT-101) in diffuse cutaneous systemic sclerosis subjects treated for one year in an open-label extension of trial jbt101-ssc-001. BMJ Publishing Group Ltd; 2018.

9

Nederlandse samenvatting

In dit proefschrift wordt de potentie van anti-nucleaire antilichamen om te functioneren als biomarker geëvalueerd. Inhoudelijk worden in dit proefschrift de heterogeniteit Systemische Sclerose (SSc), de behoefte aan biomarkers en de mogelijkheid van auto-antilichamen om als biomarker te functioneren, beschreven.

Systemische sclerose (SSc) is een complexe heterogene bindweefselziekte die een mild ziekteverloop kan hebben, maar ook levensbedreigend kan zijn (1). In de introductie wordt een ziektebeschrijving gegeven en wordt ingegaan op haar epidemiologie, historie, pathogenese, diagnosestelling en classificatie en behandelopties (**hoofdstuk 1)**. In dit hoofdstuk wordt ook een korte toelichting gegeven over het Leiden Comprehensive Care Pathway en het Combined Care in Systemic Sclerosis (CCISS) cohort, welke de basis is geweest voor al het onderzoek dat in dit proefschrift beschreven is.

De behoefte aan een biomarker

Risicostratificatie in SSc is moeilijk. Dit blijkt wel uit het ziektebeloop van de placebogroep in de RITuximab in Systemische sclerose studie (RITIS-studie) in Hoofdstuk 2. Deze studie was gericht op inclusie van patiënten met progressieve ziekte, maar bleek niet in staat die patiënten te selecteren.

De RITIS-studie was een studie waarin 16 SSc-patiënten met vroege ziekte (tijd sinds diagnose <2 jaar voorafgaand aan inclusie) 1:1 werden gerandomiseerd naar behandeling met rituximab (anti-CD20; B-celdepletie) of placebo. Aangezien bij pasgeboren muizen met een strakke huid anti-CD20 behandeling de ontwikkeling van fibrose werd voorkomen, terwijl bij volwassen muizen met een reeds strakke huid en verder gevorderde ziekte er geen effect van de behandeling was (1), werd verondersteld dat rituximab alleen een gunstig effect zou hebben in vroege ziektestadia. Helaas werden in de studie geen significante effecten waargenomen. Dit sluit echter niet uit dat sommige patiënten een gunstig effect hebben gehad van de behandeling met rituximab: Vanwege de zeldzaamheid van de ziekte is het moeilijk om grootschalige onderzoeken uit te voeren. Om het behandeleffect in een zo klein mogelijke groep te bestuderen, is selectie van een specifieke groep patiënten vereist waarin de effecten meest waarschijnlijk kunnen worden waargenomen. Voor een behandeling waarbij hypothetisch het ziekteproces gestopt wordt door de therapie en geen verbetering wordt verwacht, zou de selectie patiënten met progressieve ziekte nodig zijn. Echter, zoals blijkt uit het ziekteverloop van de placebogroep in de RITIS-studie, waarin huidscores, longfunctie en dagelijks functioneren stabiel waren, is een dergelijke selectie in SSc een uitdaging.

De RITIS-studie is niet de enige studie in SSc waarbij inclusiecriteria niet accuraat genoeg bleken om patiënten met progressieve ziekte te identificeren. Andere voorbeelden zijn de SLS I studie (2) en de Focussced-studie (3), die ook een relatief stabiel ziektebeloop laten zien in de placebogroepen. In hoofdstuk 1, tabel 1 wordt beschreven dat SSc-specifieke auto-antilichamen geassocieerd zijn met specifieke ziektekenmerken in SSc. Op basis van deze bevindingen uit cross-sectioneel onderzoek hebben verschillende auteurs gesuggereerd dat auto-antilichamen kunnen worden gebruikt om het ziektebeloop van SSc-patiënten te voorspellen (4-6). De RITIS-studie, de SLS I-studie en ook de Focussced gebruikten echter deze auto-antilichamen niet voor de selectie van patiënten in hun inclusiecriteria. In ons proefschrift hebben we daarom geprobeerd te beantwoorden of het gebruik van auto-antilichamen voor de inclusiecriteria in dergelijke onderzoeken, het onderzoeksveld zou verbeteren.

Auto-antilichamen als biomarkers bij systemische sclerose

In Hoofdstuk 3 evalueerden we de toegevoegde waarde van die auto-antilichamen voor de predictie van overleving. Hiervoor hebben we een statistische analyse uitgevoerd (hiërarchische clustering in combinatie met principale componentenanalyse), waarbij we de computer subgroepen van patiënten lieten maken op basis van respectievelijk klinische en demografische kenmerken en vervolgens dezelfde analyse uitvoerden, alleen met aanvullend gebruik van auto-antilichaamstatus. Het vergelijken van risicostratificatie met en zonder kennis van auto-antilichamen toonde aan dat het aandeel juiste voorspellingen van overleving binnen vijf jaar toenam wanneer de antilichaamsubtypes in het model werden opgenomen. Echter voor een toename van 13% in het identificeren van hoogrisico patiënten, nam het aantal te screenen patiënten toe met 27%. Dit illustreert dat hoewel auto-antilichamen geassocieerd kunnen worden met overleving, de bijdrage aan klinische prognose als het gaat om overleving beperkt is.

Van sommige SSc auto-antilichamen is beschreven dat ze geassocieerd zijn met maligniteiten. Dit is met name het geval voor RNA-polymerase III (RNApIII) (7-9)). Daarom wordt in de huidige klinische praktijk, wanneer een patiënt nieuw gediagnosticeerd wordt met RNApIII+ SSc, een maligniteitsscreening uitgevoerd. Voor andere auto-antilichamen is hun relatie met maligniteiten minder duidelijk. Bernal Bello et al. suggereerde dat Pm/Scl-antilichamen in SSc ook verband kunnen houden met een verhoogd maligniteitsrisico. In Hoofdstuk 4 laten we zien dat we deze bevinding niet konden bevestigen in het CCISS-cohort. Pathofysiologisch zou de relatie tussen SSc en kanker gebaseerd kunnen zijn op epitoop spreiding van een immuunreactie die primair gericht was op een getransformeerd oncogeen auto-antigeen. De aanwezigheid van continue ontsteking kan echter ook een situatie creëren waarin DNA-schade gemakkelijker ontstaat met de ontwikkeling van kanker als gevolg.

Concluderend bevestigen deze onderzoeken dat auto-antilichaamstatus alleen niet kan functioneren als een geschikte biomarker in SSc. De behoefte aan een biomarker is echter aanwezig, niet alleen om de juiste patiënten te selecteren voor deelname aan klinische studies, maar ook om te kunnen beslissen hoe nauwlettend de klinische follow-up moet zijn voor patiënten in de praktijk. De meeste onduidelijk is er bij patiënten met een anti-topoisomerase I auto-antilichaam. Daarom wordt voor deze groep hieronder de heterogeniteit nog verder uitgewerkt.

Anti-topoisomerase I positieve systemische sclerose

In hoofdstuk 5 hebben we de heterogeniteit van ATA-positieve SSc onderzocht. We hebben laten zien dat, zoals verwacht, ATA+-patiënten in het CCISS-cohort vaker ernstige longfibrose en diffuse huidverdikking ontwikkelen. Interessant is dat wanneer geanalyseerd wordt vanaf het tijdstip van inclusie in ons cohort, in tegenstelling tot wat men zou verwachten, er geen verschil was in ziekteprogressie en overleving tussen ATA+ en ACA+ patiënten. Ook Steen et al. hadden al in 1988 opgemerkt dat er bij analyse vanaf het begin van de ziekte een duidelijk verschil in overleving is tussen ATA+ en ACA+ patiënten, terwijl de overleving tussen ATA+ en ACA+ vergelijkbaar is vanaf hun eerste bezoek aan een gespecialiseerde SSC-kliniek (10). Met de komst van de ACR/EULAR 2013 SSc-classificatiecriteria, waarvan wordt aangenomen dat ze de diagnose van patiënten in een eerder stadium mogelijk maken (11, 12), hadden wij echter verwacht dat de huidige klinische praktijk meer in lijn zou zijn met een analyse vanaf ziekteaanvang en dus meer ziekteprogressie voor ATA+ patiënten. Uit onze analyse bleek dat dit echter niet het geval was. Het lijkt er daarmee op dat de ACR/EULAR 2013 SSc-criteria voornamelijk de diagnose van milde ziekte en niet vroege ziekte mogelijk maken. Dit werd nog eens benadrukt door het gegeven dat van alle ATA+ patiënten met een longitudinale follow-up tot 8 jaar, een derde van de patiënten nooit fibrotische complicaties ontwikkelden. Bovendien bleek het onwaarschijnlijk dat ATA+-patiënten met een normale longfunctietest bij de eerste screening, tijdens de follow-up zouden verslechteren tot ernstige longziekte. De heterogeniteit van ATA+ SSc is als zodanig duidelijk aangetoond, waarbij een groot deel van de ATA+-ziekte mild blijkt.

Bij SSc is het opmerkelijk dat hoewel de ziekte veel vaker voorkomt bij vrouwen, mannelijke patiënten vaker ATA hebben. Daarom evalueerden we de prognostische waarde van ATA+ en ACA+ afzonderlijk voor mannen en vrouwen in Hoofdstuk 6. Hierin vonden we dat geslacht niet alleen geassocieerd is met het auto-antilichaamsubtype, maar ook een onafhankelijke bijdrage levert aan de ernst van de ziekte bij SSc. Mannen hebben een grotere kans op de ontwikkeling van diffuse huidaandoeningen, pulmonale hypertensie en ziekte gerelateerde mortaliteit. Geïntensiveerde screening lijkt daarom voldoende bij alle mannelijke SSc-patiënten, onafhankelijk van de auto-antilichaamstatus.

In een poging tot betere risicostratificatie te komen bij ATA+ SSc, hebben we in Hoofdstuk 7 onderzocht of kennis van ATA isotypes kan helpen bij het identificeren van patiënten met progressieve ziekte. IgM is een isotype, waarvan bekend is dat het voorkomt in actieve fasen van veel ziekten. Onze bevinding dat ATA-IgM geassocieerd is met ziekteprogressie, bevestigde voor ons daarom dat kennis van de isotypestatus van specifieke ANA in SSc zou kunnen fungeren als aanvullende biomarker. De aanwezigheid van ATA IgM weerspiegelt waarschijnlijk de aanhoudende presentatie van voor de ziekte relevante auto-antigenen met rekrutering van kortlevende naïeve B-cellen. Maar aangezien ook een deel van de ATA-IgM+ SSc-patiënten niet achteruitgaat, is er een doorlopend onderzoek nodig naar de factoren die ten grondslag liggen aan het ontstaan van ATA-IgM+ en die verklaren waarom sommige patiënten een stabiele ziekte hebben, terwijl anderen deze levensbedreigende complicaties ontwikkelen**.**

Toekomstperspectieven op onderzoek naar systemische sclerose

Ons gebrek aan begrip van de pathofysiologie achter SSc, belemmert de ontwikkeling van succesvolle therapieën en kosteneffectieve screeningprogramma's. Naar mijn mening zou toekomstig onderzoek zich daarom moeten richten op meer begrip van exacte mechanismen die leiden tot het krijgen van de ziekte en het heterogene klinische beeld van SSc.

Een mogelijkheid om tot meer inzicht te komen, zou het bestuderen van patiënten in klinische remissie kunnen zijn. Zoals besproken in Hoofdstuk 1 zijn er drie belangrijke bijdragen aan de pathogenese van SSc: microangiopathie (13, 14), fibrose (15-18) en immunologische veranderingen (19-21). Het bestuderen van de veranderingen in deze drie compartimenten bij patiënten in klinische remissie zou wel eens de sleutel kunnen zijn tot meer inzicht in de pathofysiologie van SSc.

Een andere strategie die ons meer begrip van de onderliggende ziektemechanismen in SSc zou kunnen geven, zijn de klinische onderzoeken die wereldwijd worden uitgevoerd. Multicenter onderzoek, met inclusie van veel patiënten, heeft minder snel last van onvoldoende power. Kennis van het targets van het liganden die in klinische studies een gunstig effect laten zien, zouden wel eens een ander licht kunnen werpen op de etiologie van de ziekte. Hopelijk leiden dergelijke studies ook tot meer 'tools' voor de behandelende reumatoloog, die toch noch toe min of meer machteloos is. Voor een academisch centrum als het Leids Universitair Medisch Centrum is het daarom van onschatbare waarde om deel te kunnen nemen aan onderzoeken zoals de FASST (lanifibranor) (22) en RESOLVE (lenabasum) (23).

Biomarkers bij systemische sclerose: zijn auto-antilichamen de sterren die ons de weg wijzen?

Concluderend kan worden gesteld dat auto-antilichaam status alleen ons niet voldoende informatie geeft om risicostratificatie op een zodanige manier uit te voeren dat we de juiste patiënten voor klinische onderzoeken kunnen selecteren, danwel een op maat gemaakt screeningprogramma voor patiënten kunnen opstellen of kunnen beslissen of en welke therapie er bij een patiënt gestart zou moeten worden. Er zijn nog veel struikelblokken om deze doelen wel te bereiken. Desalniettemin weten we dat auto-antilichamen geassocieerd zijn met het fenotype van de ziekte. Daarom zouden auto-antilichamen kunnen fungeren als één van de leidende sterren in de follow-up en behandeling van SSc. We zijn echter nog op zoek naar het totaalplaatje ter ondersteuning van het navigeren. Laten we hopen dat, in tegenstelling tot ten tijde van Klee (de schilder van het werk op de omslag van dit proefschrift, dat zijn werk “Deze ster leert buigen” - 1940), we over enige tijd niet langer hoeven te buigen voor de ster van SSc, maar ook andere sterren vinden die ons helpen veilig te navigeren door de soms kalme en vredige, maar soms ook gevaarlijke en onvoorspelbare zee, wat het ziekteverloop van SSc nog steeds is.

REFERENTIES

1. Hasegawa M, Hamaguchi Y, Yanaba K, Bouaziz JD, Uchida J, Fujimoto M, et al. B-lymphocyte depletion reduces skin fibrosis and autoimmunity in the tight-skin mouse model for systemic sclerosis. Am J Pathol. 2006;169(3):954-66.
2. Tashkin DP, Elashoff R, Clements PJ, Goldin J, Roth MD, Furst DE, et al. Cyclophosphamide versus placebo in scleroderma lung disease. N Engl J Med. 2006;354(25):2655-66.
3. Khanna D, Lin CJF, Furst DE, Goldin J, Kim G, Kuwana M, et al. Tocilizumab in systemic sclerosis: a randomised, double-blind, placebo-controlled, phase 3 trial. The Lancet Respiratory medicine. 2020;8(10):963-74.
4. Nihtyanova SI, Denton CP. Autoantibodies as predictive tools in systemic sclerosis. Nat Rev Rheumatol. 2010;6(2):112-6.
5. Kuwana M. Circulating Anti-Nuclear Antibodies in Systemic Sclerosis: Utility in Diagnosis and Disease Subsetting. Journal of Nippon Medical School = Nippon Ika Daigaku zasshi. 2017;84(2):56-63.
6. Khanna D, Denton CP. Evidence-based management of rapidly progressing systemic sclerosis. Best Pract Res Clin Rheumatol. 2010;24(3):387-400.
7. Shah AA, Hummers LK, Casciola-Rosen L, Visvanathan K, Rosen A, Wigley FM. Examination of autoantibody status and clinical features associated with cancer risk and cancer-associated scleroderma. Arthritis & rheumatology. 2015;67(4):1053-61.
8. Shah AA, Rosen A, Hummers L, Wigley F, Casciola-Rosen L. Close temporal relationship between onset of cancer and scleroderma in patients with RNA polymerase I/III antibodies. Arthritis Rheum. 2010;62(9):2787-95.
9. Joseph CG, Darrah E, Shah AA, Skora AD, Casciola-Rosen LA, Wigley FM, et al. Association of the autoimmune disease scleroderma with an immunologic response to cancer. Science. 2014;343(6167):152-7.
10. Steen VD, Powell DL, Medsger TA, Jr. Clinical correlations and prognosis based on serum autoantibodies in patients with systemic sclerosis. Arthritis Rheum. 1988;31(2):196-203.
11. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/ European League against Rheumatism collaborative initiative. Arthritis Rheum. 2013;65(11):2737-47.
12. Association AR. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Arthritis Rheum. 1980;23(5):581-90.
13. Koenig M, Joyal F, Fritzler MJ, Roussin A, Abrahamowicz M, Boire G, et al. Autoantibodies and microvascular damage are independent predictive factors for the progression of Raynaud’s phenomenon to systemic sclerosis: a twenty-year prospective study of 586 patients, with validation of proposed criteria for early systemic sclerosis. Arthritis Rheum. 2008;58(12):3902-12.
14. Fleischmajer R, Perlish JS. Capillary alterations in scleroderma. J Am Acad Dermatol. 1980;2(2):161-70.
15. Manetti M, Guiducci S, Matucci-Cerinic M. The origin of the myofibroblast in fibroproliferative vasculopathy: does the endothelial cell steer the pathophysiology of systemic sclerosis? Arthritis Rheum. 2011;63(8):2164-7.

16. Kissin EY, Merkel PA, Lafyatis R. Myofibroblasts and hyalinized collagen as markers of skin disease in systemic sclerosis. Arthritis Rheum. 2006;54(11):3655-60.
17. Lafyatis R. Transforming growth factor beta-at the centre of systemic sclerosis. Nature Reviews Rheumatology. 2014;10(12):706-19.
18. Varga J, Abraham D. Systemic sclerosis: a prototypic multisystem fibrotic disorder. J Clin Invest. 2007;117(3):557-67.
19. Tan EM, Rodnan GP, Garcia I, Moroi Y, Fritzler MJ, Peebles C. Diversity of antinuclear antibodies in progressive systemic sclerosis. Anti-centromere antibody and its relationship to CREST syndrome. Arthritis Rheum. 1980;23(6):617-25.
20. Douvas AS, Achten M, Tan EM. Identification of a nuclear protein (Scl-70) as a unique target of human antinuclear antibodies in scleroderma. J Biol Chem. 1979;254(20):10514-22.
21. Sakkas LI, Xu B, Artlett CM, Lu S, Jimenez SA, Platsoucas CD. Oligoclonal T cell expansion in the skin of patients with systemic sclerosis. J Immunol. 2002;168(7):3649-59.
22. Avouac J, Konstantinova I, Guignabert C, Pezet S, Sadoine J, Guilbert T, et al. Pan-PPAR agonist IVA337 is effective in experimental lung fibrosis and pulmonary hypertension. Ann Rheum Dis. 2017:annrheumdis-2016-210821.
23. Spiera R, Hummers L, Chung L, Frech T, Domsic R, Hsu V, et al. OP0006 Safety and efficacy of lenabasum (JBT-101) in diffuse cutaneous systemic sclerosis subjects treated for one year in an open-label extension of trial jbt101-ssc-001. BMJ Publishing Group Ltd; 2018.

Appendices

Curriculum Vitae

Maaike Boonstra werd op 16 december 1987 geboren te Lelystad. In 2006 behaalde zij haar diploma Voortgezet Wetenschappelijk Onderwijs aan de Interconfessionele Scholengemeenschap Arcus te Lelystad, waarna zij enkele jaren Farmaceutische Wetenschappen studeerde aan de Vrije Universiteit te Amsterdam. In 2008 begon zij met de studie Geneeskunde aan de Vrije Universiteit te Amsterdam, waar zij in 2012 haar bachelorsdiploma haalde. Tijdens de master Geneeskunde, volgde zij tussen september 2011 en december 2012 verschillende vakken Rechtsgeleerdheid aan de Universiteit van Amsterdam. In september 2015 rondde zij haar master Geneeskunde af met een wetenschappelijk onderzoek naar contacteczeem bij kinderen en volwassenen met atopisch eczeem, waarbij zij verbonden was aan zowel het Academisch Medisch Centrum te Amsterdam (begeleider: dr. M.A. Middelkamp Hup) als VU medisch centrum (begeleider: prof. dr. T. Rustemeyer).

In januari 2016 startte zij met haar promotieonderzoek op de afdeling reumatologie van het Leids Universitair Medisch Centrum onder begeleiding van prof. dr. T.W.J. Huizinga en dr. J.K. de Vries-Bouwstra. Tijdens dit promotieonderzoek werden verschillende onderzoeken verricht, met behulp van gegevens verzameld in het kader van het zorgpad Sclerodermie van het Leids Universitair Medisch Centrum, waarvan de resultaten te vinden zijn in deze thesis.

Sinds eind december 2018 is zij als arts in opleiding tot reumatoloog verbonden aan het Amsterdam Universitair Medisch Centrum (opleider: prof. dr. W.F. Lems). De vooropleiding interne geneeskunde werd gevolgd in het VU medisch centrum (opleider: Y.M. Smulders) en Spaarne Gasthuis (opleider: W. de Ronde). Momenteel volgt zij haar perifere stage in het Spaarne Gasthuis (opleider: dr. S. ten Wolde).

LIST OF PUBLICATIONS

FIRST AUTHOR

Rheumatology

Rituximab in early systemic sclerosis
M. Boonstra, J. Meijs, A.L. Dorjée, N. Ajmone Marsan, A. Schouffoer M.K. Ninaber, K.D. Quint, F. Bonte-Mineur, T.W.J. Huizinga, H.U. Scherer, J.K. de Vries-Bouwstra
RMD Open. 2017 Jul 28; 3

Auto-antibodies and cancer in systemic sclerosis
M. Boonstra, J.K. de Vries-Bouwstra, T.W.J. Huizinga
Autoimmun Rev. 2017 Aug; 16(8):883-884

To what extent do auto-antibodies help to identify high-risk patients in systemic sclerosis?
M. Boonstra, B.J.A. Mertens, J.A. Bakker, M.K. Ninaber, N. Ajmone Marsan, A.H.M van der Helm-van Mil, H.U. Scherer, T.W.J. Huizinga, J.K. de Vries-Bouwstra
Clin Exp Rheumatol. 2018 Jul 18

Prognostic properties of anti-topoisomerase antibodies in patients identified by the ACR/EULAR 2013 systemic sclerosis criteria
M. Boonstra, M.K. Ninaber, N. Ajmone Marsan, T.W.J. Huizinga, H.U. Scherer, J.K. de Vries-Bouwstra
Rheumatology (Oxford). 2019 Apr 1;58(4):730-732

Association of Anti-Topoisomerase I Antibodies of the IgM Isotype With Disease Progression in Anti-Topoisomerase I-Positive Systemic Sclerosis
M. Boonstra, J.A. Bakker, A. Grummels, M.K. Ninaber1, N. Ajmone Marsan, C.M. Wortel, T.W.J. Huizinga, S. Jordan, A.M. Hoffman-Vold, O. Distler, R.E.M. Toes, H.U. Scherer, J.K. de Vries-Bouwstra
Arthritis Rheumatol. 2020 Nov;72(11):1897-1904.

The effect of sex on outcomes in systemic sclerosis: does anti-topoisomerase I status matter? A EUSTAR analysis
M. Boonstra, S. le Cessie, A. Riccardi, P. Airo, O. Distler, M. Matucci-Cerinic, C. Caimmi, E. Siegert, Y. Allanore, T.W.J. Huizinga, R.E.M. Toes, H.U. Scherer, Jeska K de Vries-Bouwstra, EUSTAR collaborators
– submitted -

Dermatology

Both children and adult patients with difficult-to-treat atopic dermatitis have high prevalences of concomitant allergic contact dermatitis
M. Boonstra, T. Rustemeyer, M. A. Middelkamp Hup
J Eur Acad Dermatol Venereol. 2018 Sep;32(9):1554-1561

Unilaterale naevoïde teleangiëctasieën
M. Boonstra, L.A. Gongrijp
Ned Tijdschr Dermatol Venereol 2015 Sep; 25(8):415-417

CO-AUTHOR

Antisense long non-coding RNAs are deregulated in skin tissue of patients with systemic sclerosis
T.C. Messemaker, L. Chadli, G.,Cai, V.S. Goelela, M. Boonstra, A.L. Dorjée, S.N. Andersen, H.M.M. Mikkers, P. van 't Hof, H. Mei, O. Distler, H.H.M. Draisma, M.E. Johnson, N.M. Orzechowski, R.W. Simms, R.E.M. Toes, J. Aarbiou, T.W.J. Huizinga, M.L. Whitfield, J. DeGroot, J.K. de Vries-Bouwstra, F. Kurreeman
J Invest Dermatol. 2017 Nov 24

Clinical and echocardiographic associates of all-cause mortality and cardiovascular outcomes in patients with systemic sclerosis
S.E. van Wijngaarden, M. Boonstra, B. Bloem, D. Cassani, F.C. Tanner, S. Jordan, O. Distler, V. Delgado, J.J. Bax, J.K. de Vries-Bouwstra, N. Ajmone Marsan
JACC Cardiovasc Imaging. 2019 Nov; 12(11):2273-2276

Illness perceptions, risk perceptions and worries in patients with early systemic sclerosis: A focus group study
N.M. van Leeuwen, M. Boonstra, T.W.J. Huizinga, A.A. Kaptein, J.K. de Vries-Bouwstra
Musculoskeletal Care. 2020 Jun;18(2):177-186

Degree of Vasculopathy in Systemic Sclerosis Patients with Anti-U3RNP Antibody Indicates Need for Extensive Cardiopulmonary Screening
N.M. van Leeuwen, M. Boonstra, N. Ajmone Marsan, M.K. Ninaber, T.W.J. Huizinga, J.K. de Vries-Bouwstra
J Rheumatol, 2019 Sep;46(9):1244-1245

Predictive factors for treatment-related mortality and major adverse events after autologous haematopoietic stem cell transplantation for systemic sclerosis: results of a long-term follow-up multicentre study
S. van Bijnen, J.K. de Vries-Bouwstra, C.H. van den Ende, M. Boonstra, L. Kroft, B. Geurts, M. Snoeren, A. Schouffoer, J. Spierings, J.M. van Laar, T.W.J. Huizinga, A. Voskuyl, E. Marijt, W. van der Velden, F.H.J. van den Hoogen, M.C. Vonk
Ann Rheum Dis. 2020 Aug;79(8):1084-1089

Evolution of interstitial lung disease one year after hematopoietic stem cell transplantation or cyclophosphamide for systemic sclerosis
J. Ciaffi, N.M. van Leeuwen, M. Boonstra, L.J.M. Kroft, A.A. Schouffoer, M.K. Ninaber, T.W.J. Huizinga, J.K. de Vries-Bouwstra
2022 Mar;74(3):433-441

Anticentromere Antibody Levels and Isotypes and the Development of Systemic Sclerosis
N.M. van Leeuwen, M. Boonstra, J.A. Bakker, A. Grummels, S. Jordan, S. Liem, O. Distler, A. Hoffmann-Vold, K. Melsens, V. Smith, M. Truchetet, H.U. Scherer,R. Toes, T.W.J. Huizinga, J.K. de Vries-Bouwstra
Arthritis Rheumatol. 2021 Dec;73(12):2338-2347

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