To what extent do autoantibodies help to identify high-risk patients in systemic sclerosis?

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ABSTRACT

Objective. *To evaluate the additive value of autoantibodies in identifying systemic sclerosis (SSc) patients with high complication risk.*

Methods. *Patients entering the Combined Care In SSc cohort, Leiden University Medical Centre 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 autoantibody 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 autoantibodies 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 autoantibody 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. *Autoantibodies 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), gastrointestinal 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 sub-classification 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 autoantibodies 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 autoantibody status, possibly of help in risk-stratification. Currently, autoantibody status is considered to be 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 autoantibody status, the actual contribution of the autoantibody to risk-stratification is partially blurred. Knowledge of the specific contribution of the autoantibody can on the one hand improve clinical risk-stratification, and on the other hand shed light on the actual pathophysiological role of the autoantibody 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 autoantibody 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 catheterisation/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), diarrhoea (yes/no), intestinal distension (yes/no), constipation (yes/no), faecal incontinence (yes/no), parenteral nutrition (yes/no), history of gastric antral vascular ectasia (GAVE) (yes/no); 8) laboratory findings: CRP level, haemoglobin (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.

Autoantibody testing

In a previous study (15), extensive autoantibody 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 (autoantigen 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 immuno assay (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 standardising (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% 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

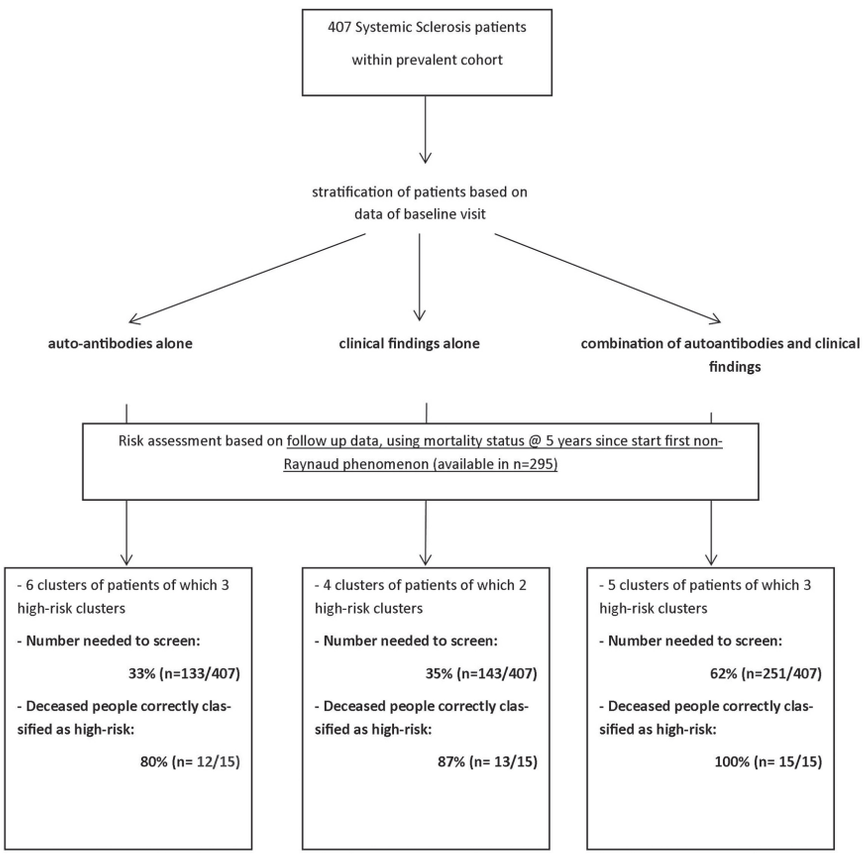

Fig. 1. Study flow chart. Risk assessment based on subsetting of patients according to auto-antibodies alone, clinical findings alone or the combination of auto-antibodies and clinical findings.

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.

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.

This process was performed using demographic and clinical variables, excluding autoantibody and survival data. Next, this process was repeated, with additional inclusion of 6 variables for disease-specific autoantibody 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 I. Of 407 patients included, data on autoantibody 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). 23% (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.

Autoantibody 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 autoantibodies 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 (Fig. 2). The clinical phenotype of the patients in these 4 subgroups is shown in Table II.

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%, $p<0.001$),

Table I. Baseline characteristics of patients with specific autoantibodies 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)
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
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)
diarrhoea, % 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)
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-fibrillarine; 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.

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% ($p<0.001$).

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], diarrhoea 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

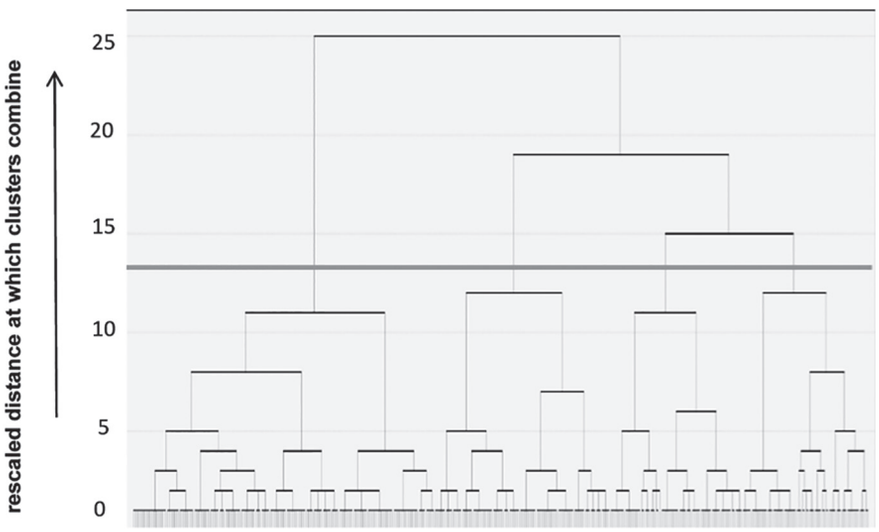

Fig. 2. Dendrogram of cluster analysis of systemic sclerosis patients using solely clinical variables. Cluster process was done by Wards method, using Euclidean distance on standardised variables (range -1 to 1) of scores on the first 7 factors obtained by principal component analysis on 52 clinical 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.

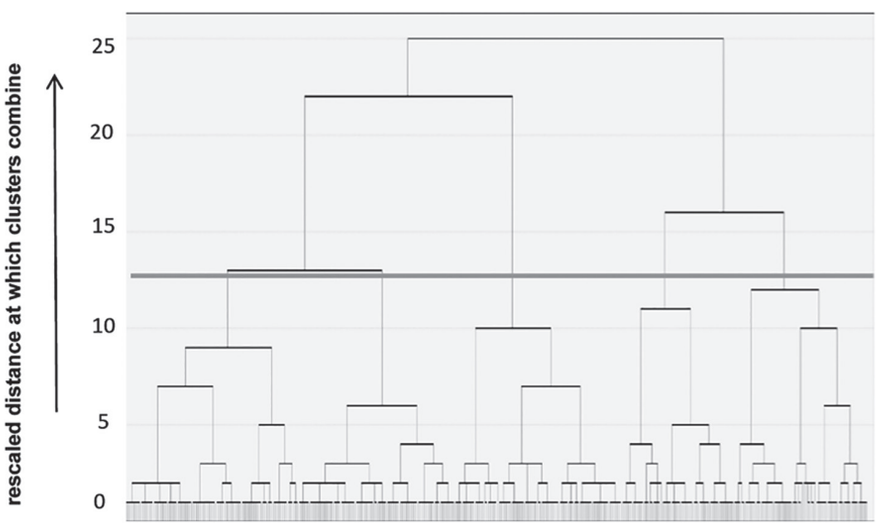

Fig. 3. Dendrogram of cluster analysis of systemic sclerosis patients using clinical variables and auto-antibodies. Cluster process was done by Wards method, using Euclidean distance on standardised variables (range -1 to 1) of scores on the first 7 factors obtained by principal component analysis on 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.

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], diarrhoea 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 autoantibodies were not taken into account in the subgroup process, autoantibodies 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 autoantibody in subgroups 3 (55% [n=53], p<0.001) and 4 (45% [n=74], p=0.032). Prevalences of RNApIII, 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.

Stratification of patients based on clinical variables and disease-specific autoantibodies

Using clinical variables and additionally, autoantibody 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 (Fig. 3). Clinical characteristics of the patients in the different subgroups are shown in Table III.

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 characterised by a high frequency of GI involvement and subgroup 4 represented a miscellaneous subgroup. The additional subgroup 5 was characterised by less frequent ILD (mean predicted FVC 113%, p<0.001; mean predicted DLCO 69.9 p<0.001), low TR

Table II. 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								
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
diarrhoea, % of patients (n)	4.3 (3)	**0.004**	12.3 (9)	0.328	35.1 (34)	**<0.001**	9.0 (15)	**0.015**
Renal								
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 I.
**mortality in patients with available follow-up data of at least 5 years since first non-Raynaud's phenomenon. (subgroup 1 n=53; subgroup 2 n=57; subgroup 3 n=78; subgroup 4 n=104).

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 autoantibodies, 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 autoantibody (37%, p=0.473). This subgroup was additionally characterised by a high prevalence of RNApIII autoantibodies (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 autoantibody status alone, stratification based

Table III. Clinical characteristics and autoantibody prevalences within systemic sclerosis subgroups obtained by cluster analysis using clinical variables and disease specific autoantibody 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
duration of scleroderma (yr.) 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**
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
diarrhoea, % 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
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 I.
**mortality in patients with available follow-up data of at least 5 years since first non-Raynaud's phenomenon. (subgroup 1 n=60; subgroup 2 n=44; subgroup 3 n=70; subgroup 4 n=52; subgroup 5 n=69).

on clinical variables and stratification based on both clinical variables and autoantibody status. Based on autoantibody 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 autoantibodies 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 dif-

ferent subgroups, either using auto-antibodies, clinical or combined data for stratification.

Discussion

With this study we aimed at assessing the additional value of autoantibodies as markers for severe disease course in SSc, in the clinical setting. We show that when autoantibodies 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 autoantibody 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 III).

Based on these findings we conclude that estimating prognosis for the individual patient based on autoantibody status alone, as is suggested for early disease (7, 11), is imprecise and as such inappropriate. Conform to 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*. and Cottrell *et al*. demonstrate that prognosis cannot solely be estimated based on autoantibody status, but assessment of clinical features such as skin is meaningful (10, 17).

Nevertheless, autoantibodies 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 autoantibody status, we hypothesise that other autoantibody characteristics are of relevance for autoantibody pathogenicity as has been described in other autoimmune 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 autoantibody characteristics such as fine-specificity, isotype prevalences, Fc-glycosylation and titre 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 titres 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 relative 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 autoantibodies 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 autoantibody status and show that autoantibodies are of additional value in risk-stratification and clinical subsetting in SSc. This underlines the hypothesis that autoantibodies 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 autoantibodies 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.

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