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Interpretation of an Extended Autoantibody Profile in a Well-Characterized Australian Systemic Sclerosis (Scleroderma) Cohort Using Principal Components Analysis

K. A. Patterson,[1] P. J. Roberts-Thomson,[2] S. Lester,[3] J. A. Tan,[4] P. Hakendorf,[4] M. Rischmueller,[5] J. Zochling,[6] J. Sahhar,[7] P. Nash,[8] J. Roddy,[9] C. Hill,[5] M. Nikpour,[10] W. Stevens,[11] S. M. Proudman,[12] and J. G. Walker[13]

Objective. **To determine the relationships between systemic sclerosis (SSc)–related autoantibodies, as well as their clinical associations, in a well-characterized Australian patient cohort.**

Methods. **Serum from 505 Australian SSc patients were analyzed with a commercial line immunoassay (EuroLine; Euroimmun) for autoantibodies to centromere proteins CENP-A and CENP-B, RNA polymerase III (RNAP III; epitopes 11 and 155), the 90-kd nucleolar protein NOR-90, fibrillarin, Th/To, PM/Scl-75, PM/Scl-100, Ku, topoisomerase I (topo I), tripartite motif–containing protein 21/Ro 52, and platelet-derived growth factor receptor. Patient subgroups were identified by hierarchical clustering of the first 2 dimensions of a principal components analysis of quantitative autoantibody scores. Results were compared with detailed clinical data.**

Results. **A total of 449 of the 505 patients were positive for at least 1 autoantibody by immunoblotting. Heatmap visualization of autoantibody scores, along with principal components analysis clustering, demonstrated strong, mutually exclusive relationships between CENP, RNAP III, and topo I. Five patient clusters were identified: CENP, RNAP III strong, RNAP III weak, topo I, and other. Clinical features associated with CENP, RNAP III, and topo I were consistent with previously published reports concerning limited cutaneous and diffuse cutaneous SSc. A novel finding was the statistical separation of RNAP III into 2 clusters. Patients in the RNAP III strong cluster had an increased risk of gastric antral vascular ectasia, but a lower risk of esophageal dysmotility. Patients in the other cluster were more likely to be male and to have a history of smoking and a history of malignancy, but were less likely to have telangiectasia, Raynaud's phenomenon, and joint contractures.**

Conclusion. **Five major autoantibody clusters with specific clinical and serologic associations were identified in Australian SSc patients. Subclassification and disease stratification using autoantibodies may have clinical utility, particularly in early disease.**

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[1]K. A. Patterson, BMedSc, BSci (Hons): Flinders University, Bedford Park, South Australia, and Commonwealth Scientific and Industrial Research Organization (CSIRO), Adelaide, South Australia, Australia; [2]P. J. Roberts-Thomson, MD, DPhil (Oxon): Flinders University and Flinders Medical Centre, Bedford Park, South Australia, and SA Pathology, Adelaide, South Australia, Australia; [3]S. Lester, BSc (Hons): Queen Elizabeth Hospital, Woodville, South Australia, Australia; [4]J. A. Tan, MRCP, FRACP, P. Hakendorf, BSc, MPH: Flinders Medical Centre, Bedford Park, South Australia, Australia; [5]M. Rischmueller, MBBS, FRACP, C. Hill, MBBS, MD, FRACP: University of Adelaide, Adelaide, South Australia, and Queen Elizabeth Hospital, Woodville, South Australia, Australia; [6]J. Zochling, BMedSc, MBBS, FRACP, MMed (ClinEpi), PhD: Menzies Institute for Medical Research, Hobart, Tasmania, Australia; [7]J. Sahhar, MBBS (Hons), FRACP: Monash Health and Monash University, Melbourne, Victoria, Australia; [8]P. Nash, MBBS (Hons), FRACP: University of Queensland, Brisbane, Queensland, Australia; [9]J. Roddy, MBBS, FRACP: Royal Perth Hospital, Perth, Western Australia, Australia; [10]M. Nikpour, MBBS, PhD: University of Melbourne and St. Vincent's Hospital Melbourne, Melbourne, Victoria, Australia; [11]W. Stevens, MBBS, FRACP: St. Vincent's Hospital, Melbourne, Victoria, Australia; [12]S. M. Proudman, MBBS (Hons), FRACP: University of Adelaide and Royal Adelaide Hospital, Adelaide, South Australia, Australia; [13]J. G. Walker, MBBS, FRACP, PhD: Flinders University and Flinders Medical Centre, Bedford Park, South Australia, and Repatriation General Hospital, Daw Park, South Australia, Australia.

Address correspondence to J. G. Walker, MBBS, FRACP, PhD, Immunology, Allergy, and Arthritis Department, Flinders University, GPO Box 2100, Adelaide, South Australia 5000, Australia. E-mail: Jenny.Walker@health.sa.gov.au.

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Systemic sclerosis (SSc) is a heterogeneous disorder with well-documented genetic (1–4) and geographic (5) variations. Historically, stratification of patients with early disease has been difficult due to the absence of definitive laboratory markers and the problems inherent with a purely clinical subclassification in a condition that fluctuates with time. In 1980, the American College of Rheumatology (ACR) preliminary criteria for the classification of SSc were published (6). Since then, there has been continuing discussion about whether this classification tool should be expanded to include all patients with variant subsets (7) whose conditions fall within the scleroderma spectrum disorders (8,9). Initial validation studies of the ACR/European League Against Rheumatism (EULAR) 2013 classification criteria for SSc (10) showed improved accuracy, but this criteria set does not encompass subclassification nor does it provide prognostic information. Improved biomarkers for the subclassification of SSc subsets are sorely needed.

Serum autoantibodies are found in as many as 95% of patients with SSc (11) and carry greater weight in the newly proposed ACR/EULAR classification system. CENP, topoisomerase I (topo I), and RNA polymerase III (RNAP III) closely reflect patterns of organ involvement and disease progression (11,12), and with rare exception, they are mutually exclusive. Other serum autoantibodies are known to be associated with SSc (13,14), but their clinical associations and frequencies are less well defined. Until recently, many of these rarer autoantibodies were only available in the research setting and required labor-intensive laboratory analyses. The advent of new, less labor-intensive technologies, such as the line immunoassay, means that multiple autoantibodies, including some rarer ones, can now be tested in routine clinical practice. The clinical significance of these rarer autoantibodies remains uncertain, as does the significance of positivity for multiple autoantibodies.

We report herein on the clinical and serologic associations of 10 autoantibodies in a large, well-characterized, multicenter study of SSc patients whose sera were tested with a line immunoassay. The results demonstrate that the use of an extended autoantibody provides useful prognostic information at the time of diagnosis, and they confirm the utility of autoantibodies in the SSc spectrum of disorders. Autoantibody profiling will, we predict, have added potential in allowing the subclassification and stratification of patients in clinical trials.

PATIENTS AND METHODS

Study design. This was a cross-sectional study of a cohort of Australian SSc patients. The study was approved by the ethics committee of the participating centers and was carried out according to the 2007 National Statement on Ethical Conduct in Research involving Humans, which was published by the National Health and Medical Research Council of Australia (15), and in accordance with the Declaration of Helsinki (16).

Patient population. Patients were drawn from the Australia Scleroderma Cohort Study (ASCS), a prospective study initiated in 2007 that was designed to assess risk factors for outcomes in SSc. Patients were recruited from 8 Australian centers specializing in the care of patients with SSc: St. Vincent's Hospital and Monash Health (Melbourne, Victoria), Sunshine Coast Rheumatology and Prince Charles Hospital (Brisbane, Queensland), Royal Adelaide Hospital (Adelaide, South Australia), St. George Hospital (Sydney, New South Wales), Royal Perth Hospital (Perth, Western Australia), and Royal Hobart Hospital (Hobart, Tasmania).

ASCS participants are assessed annually, and for this analysis, data were censored on June 7, 2013. At the time of testing, there were 1,139 enrolled patients, and samples from 505 were available from the ASCS Serum Repository (Adelaide, South Australia, Australia). Patients were admitted to this database based on 1 of the following 3 salient conditions: 1) the patient fulfilled the ACR 1980 criteria (454 of 505), 2) the patient fulfilled the LeRoy and Medsger criteria for early SSc (46 of 505) (17), or the condition was diagnosed according to the expert opinion of the patient's attending physician (15 of 505).

Data collected include disease classification, sex, ethnicity, antinuclear antibody pattern by immunofluorescence, age at onset of Raynaud's phenomenon, and age at onset of first non–Raynaud's phenomenon symptom of SSc. Disease manifestations were defined as present if they had occurred since the time of diagnosis, except for the highest modified Rodnan skin thickness score (MRSS), which was since the time of enrollment in the ASCS. Variables collected included smoking history, Raynaud's phenomenon, digital ulcers, digital gangrene, digital amputation, nailfold capillary dilatation, telangiectasia, calcinosis, tendon friction rubs, joint contractures, highest MRSS, gastric antral vascular ectasia (GAVE), esophageal dysmotility, esophageal reflux, anal incontinence, bowel dysmotility, systemic hypertension, pulmonary arterial hypertension (PAH), interstitial lung disease (ILD), renal transplant, renal crisis, renal involvement, malignancy, synovitis, myositis, highest ever erythrocyte sedimentation rate, highest ever serum creatine kinase, lowest ever estimated glomerular filtration rate, C3 and C4 complement levels, and electrocardiogram abnormalities.

The clinical variables have been defined in greater detail elsewhere (18), including the specifics in this cohort of PAH, ILD, and renal crisis (19). Of note, PAH was defined as a mean pulmonary artery pressure of $\geq$25 mm Hg and a pulmonary capillary wedge pressure of $\leq$15 mm Hg on right-sided heart catheterization. ILD was defined as the presence of pulmonary fibrosis on lung imaging, usually by high-resolution computed tomography scan. Disease onset was defined as the age at onset of the first non–Raynaud's phenomenon manifestation of SSc.

Autoantibody analysis. Sera were analyzed using a commercially available line immunoblot assay (Systemic Sclerosis [Nucleoli] Profile EuroLine [IgG]; Euroimmun), and analysis was performed in a single laboratory by a single operator (KAP). Serum aliquots were stored at -80°C until the time of testing. The assay was performed according to the manufac-

turer's instructions. The kit contains 13 recombinant antigens: those expressed in *Escherichia coli* (RNA polymerase III [RNAP III; subunits RP11 and RP155], fibrillarin, the 90-kd nucleolar protein NOR-90, and Th/To) or in insect cells using the baculovirus system (CENP-A, CENP-B, PM/Scl-100, PM/Scl-75, Ku, and tripartite motif–containing protein 21 [TRIM-21]/Ro 52) plus PDGFR expressed in mammalian cells and native topo I (Scl-70) isolated from calf and rabbit thymus.

Sera were analyzed at a dilution of 1:101, and autoantibodies were detected using alkaline phosphatase–labeled anti-human IgG. The EuroLine flatbed scanner provides semiquantitative results. Readings obtained with a signal intensity of +, ++, and +++ were defined as positive and were allocated scores that equated to +1, +2, and +3, respectively. Borderline signals or no signal, representing signal intensities of <11 intensity units, were defined as negative and were scored as 0. All scores were then used in the principal components analysis. Separate studies have found good correlation between the signal intensity in the immunoblot assay and autoantibody titers/concentrations measured in alternative assays (20–22).

Statistical analysis. To enable the reduction of multicollinear data as a means of exploring underlying structures, the data were examined by principal components analysis of the autoantibody scores as performed in the R Library FactoMineR (23). Examination of the correlations between each autoantibody by the principal components analysis dimensions (Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39316/abstract) revealed that only dimensions 1 and 2 captured both positive and negative relationships between different autoantibodies, and therefore, these 2 dimensions were subsequently used for hierarchical clustering of SSc patients into autoantibody-defined subgroups.

Clinical associations with these autoantibody clusters were explored using the v test function in the FactoMineR library, which compares each group mean to the overall mean. Fisher's exact test was also performed for clinical associations when the number of patients was small. Further demographic and phenotypic data were analyzed using IBM SPSS Statistics software version 22.0.

RESULTS

Characteristics of the study patients. Demographic, clinical, and serologic characteristics of the 505 SSc study patients are presented in Table 1. At the time of entry into the ASCS, patients in this cohort were judged to have SSc according to the ACR 1980 criteria, Medsger criteria, or expert opinion. At the time of data censorship for this study, the 2013 ACR/EULAR criteria were not yet published; however, retrospective analysis showed that 498 of the 505 patients (98%) fulfilled this new set of criteria.

Findings of the autoantibody analysis. *Frequency and combination.* Counts of individual autoantibodies and their expression, either monospecifically or the total number of times they appeared with other autoantibodies, are shown in Table 2 and in Supplementary Figure 1 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39316/abstract). A total of 225 patients (45%) had a monospecific autoantibody, while 165 (33%), 49 (9%), and 10 (2%) patients were positive for 2, 3, or more autoantibodies, respectively. The remaining 56 patients (11%) were negative for all autoantibodies by immunoblot analysis (Supplementary Figure 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39316/abstract).

The majority of patients were positive for 1 of 3 major autoantibodies: CENP, topo I, or RNAP III, and coexpression of autoantibodies was common (Table 2). TRIM-21/Ro 52 was the most frequent autoantibody that occurred in combination with other autoantibodies, and only 19 patients (3.76%) were monospecific for TRIM-21/Ro 52. Topo I was observed in 112 patients but was the sole autoantibody in only 68 (61%) and was the most frequent monospecific autoantibody, and PDGFR was the least frequent monospecific autoantibody. Coexpression of CENP and topo I occurred rarely (15 of 505).

Table 1. Demographic, clinical, and serologic characteristics of the 505 SSc patients from the ASCS*

Sex	
No. (%) female	443 (87.7)
No. (%) male	62 (12.3)
Female-to-male ratio	7:1
Age, mean ± SD years	63.31 ± 12.31
At onset of RP	41.4 ± 15.77
At onset of first non-RP symptom of SSc	46.01 ± 14.06
Disease duration, mean ± SD years	11.96 ± 9.97
Ethnicity, no. (%)	
Caucasian	462 (91.5)
Asian	19 (3.8)
Aboriginal/Torres Strait Islander	8 (1.6)
Hispanic	1 (0.1)
Data not recorded	15 (3)
Disease classification, no. (%)	
Diffuse	135 (26.7)
Limited	370 (73.3)
Criteria fulfilled	
ACR/EULAR 2013 criteria	498 (98)
ACR 1980 criteria	454 (89.9)
Antinuclear antibody profile, no. (%)	
Positive	476 (94.3)
Staining pattern	
Nucleolar	125 (24.8)
Speckled	138 (27.3)
Centromere	212 (42)
Homogeneous	113 (22.4)

* Systemic sclerosis (SSc) patients were from the Australia Scleroderma Cohort Study (ASCS). Data were available from 490 patients for age at onset of Raynaud's phenomenon (RP) and from 498 patients for age at onset of non-RP symptoms and for disease duration. ACR = American College of Rheumatology; EULAR = European League Against Rheumatism.

Table 2. Numbers and combinations of autoantibodies identified in the 505 SSc patients from the ASCS*

Autoantibody	TRIM-21/Ro 52	Ku	PM/Scl	Th/To	NOR-90	Fibrillarin	RNAP III	CENP	Topo I	PDGFR
TRIM-21/Ro 52	19	5	8	8	7	3	25	102	26	1
Ku	–	3	0	0	0	3	3	5	1	0
PM/Scl	–	–	11	4	4	1	14	51	18	0
Th/To	–	–	–	3	1	0	1	4	0	0
NOR-90	–	–	–	–	1	0	5	11	1	0
Fibrillarin	–	–	–	–	–	1	2	0	1	0
RNAP III	–	–	–	–	–	–	28	13	3	0
CENP	–	–	–	–	–	–	–	86	15	1
Topo I	–	–	–	–	–	–	–	–	68	1
PDGFR	–	–	–	–	–	–	–	–	–	–
No. of monospecific autoantibodies†	19	3	11	3	1	1	28	86	68	0
Total no. of patients‡	178	14	89	15	19	6	81	228	112	2

* Systemic sclerosis (SSc) patients were from the Australia Scleroderma Cohort Study (ASCS). TRIM-21 = tripartite motif–containing protein 21; RNAP III = RNA polymerase III; topo I = topoisomerase I; PDGFR = platelet-derived growth factor receptor.
† Monospecific expression of autoantibody without any other autoantibody coexpression.
‡ Total numbers of patients with expression of each autoantibody. Due to multiple coexpression (2 or more autoantibodies in a given patient), these numbers may be less than the sum for the given autoantibody.

Cluster analysis. Strong negative associations between the 3 major autoantibodies were evident (Figure 1A), with principal components analysis of autoantibodies revealing 5 major clusters (Figure 1B): CENP, RNAP III strong, RNAP III weak, topo I, and other. Clustering was determined by the presence of 1 of the 3 major autoantibodies and 1 further group that was positive for 1 or more of the other autoantibodies tested.

Heatmap of immunoblot scores. The association between the presence and staining intensity of specific

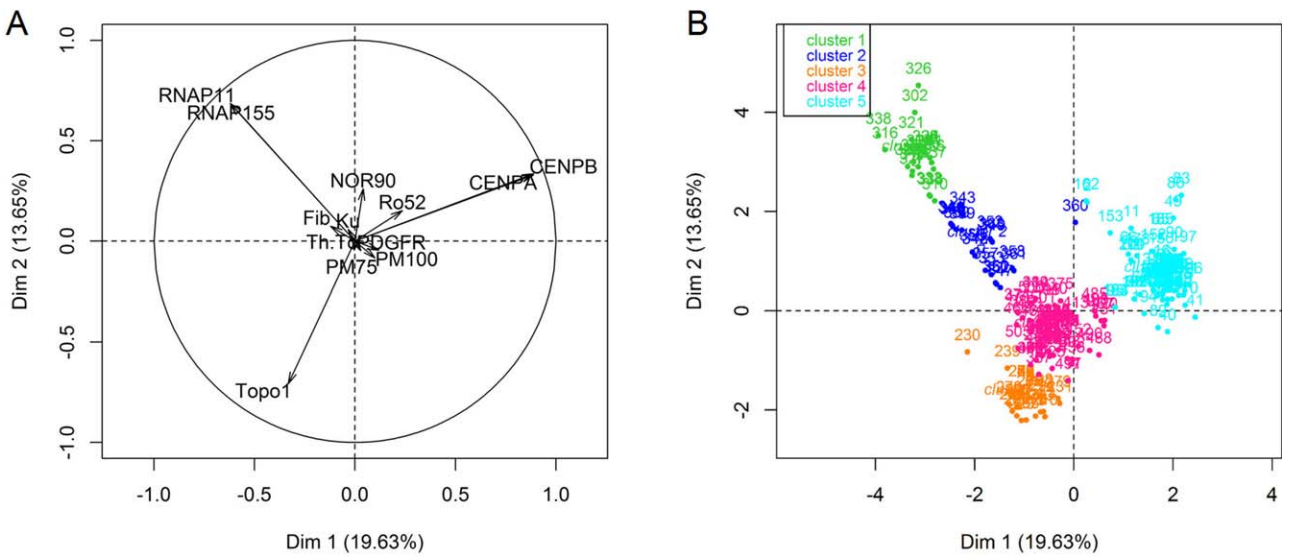

Figure 1. Principal components analysis and hierarchical clustering of immunoblot assay autoantibody scores (range 0–3) in 505 patients with systemic sclerosis. **A,** Correlation circle plot of the first 2 dimensions (Dim 1 and Dim 2) of the principal components analysis, which accounted for 51.4% of the total variance. This plot illustrates strong correlations between RNA polymerase III (RNAP III) epitope 11 (RNAP11) and RNAP III epitope 155 as well as between CENP epitopes A and B. The mutually exclusive relationships between RNAP III, CENP, and topoisomerase I (topo I) are illustrated by the equidistant arrows. There is a modest correlation between tripartite motif–containing protein 21 (TRIM-21)/Ro 52 and CENP. **B,** Hierarchical clustering of the first 2 dimensions by principal components analysis, which resulted in 5 autoantibody clusters: RNAP III strong positivity (39 patients [8%]; cluster 1), RNAP III weak positivity (25 patients [5%]; cluster 2), topo I (93 patients [18%]; cluster 3), other (142 patients [28%]; cluster 4), and CENP (206 patients [41%]; cluster 5). Fib = fibrillarin; PDGFR = platelet-derived growth factor receptor.

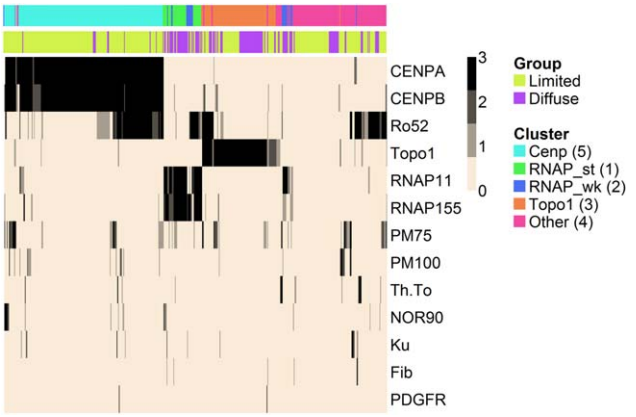

Figure 2. Heatmap of the immunoblot assay autoantibody scores in 505 patients with systemic sclerosis (SSc). Individual patients are represented by the columns, and individual autoantibodies are represented by the rows. The annotation bars at the top designate the autoantibody cluster allocation for each patient (as described in Figure 1B) and the limited or diffuse classification of each patient's SSc. See Figure 1 for other definitions.

autoantibodies as compared with the dichotomized classification of limited cutaneous SSc and diffuse cutaneous SSc. The pattern of organization of the clusters is demonstrated in a color-coded heatmap (Figure 2).

Clinical associations of groups identified by cluster analysis. The 5 SSc clusters identified by principal components analysis were assessed with regard to their clinical characteristics. The results are shown in Table 3.

Cluster CENP revealed many features consistent with limited cutaneous SSc. Clusters associated with RNAP III were statistically separated between strong and weak positive responses. Both cluster RNAP III strong and cluster RNAP III weak demonstrated multiple features of diffuse cutaneous SSc, as did cluster topo I. Cluster other was inversely associated with the presence of Raynaud's phenomenon, telangiectasia, and joint contractures and was positively associated with male sex, a history of smoking, and a history of malignancy.

Division of cluster 2 RNAP III strong and cluster 3 RNAP III weak. Cluster RNAP III weak had a weaker reactivity with RNAP III, either as a result of weaker scores or because it was reactive with only 1 epitope.

Table 3. Clinical manifestations with cluster association in SSc*

Demographic or clinical variable	All SSc patients (n = 505)	Cluster					Global *P*
		CENP (n = 206)	RNAP III strong (n = 39)	RNAP III weak (n = 25)	Topo I (n = 93)	Other (n = 142)	
Diffuse SSc	27	5†	74†	68†	51†	23	<0.001
Digital ulcers	51	46‡	64	56	64§	46	0.016
Joint contractures	46	36†	82†	68‡	62†	37§	<0.001
Raynaud's phenomenon	95	97	97	92	97	90§	0.037¶
Telangiectasia	90	94§	92	100	87	84§	0.007¶
History of smoking	47	47	41	48	37‡	56‡	0.064
Malignancy	13	8	16	8	12	19§	0.039¶
Male sex	12	5†	10	4	18	20†	<0.001
Age at onset	46	48‡	47	46	43‡	45	0.13
Pulmonary arterial hypertension	12	17§	13	12	4§	10	0.031¶
Calcinosis	43	49‡	53	32	32‡	40	0.039
Reflux esophagitis	56	63§	56	60	51	49	0.088
Anal incontinence	31	45†	28	24	17†	23	<0.001
Dry eyes	67	73‡	56	52	65	66	0.092
Dry mouth	77	84§	79	60	73	72	0.008
Renal crisis	3	0	18†	20§	1	0	<0.001¶
Systemic hypertension	53	51	74§	68	44	54	0.014
Tendon friction rubs	13	8§	21	24	14	15	0.055¶
GAVE	8	7	41†	12	1	4	<0.001¶#
Esophageal dysmotility	12	13	0§	16	8	15	0.053¶#
Renal transplant	1	0	0	8‡	0	1	0.006¶
Interstitial lung disease	33	15†	36	32	74†	33	<0.001

* The systemic sclerosis (SSc) patients were allocated objectively into autoantibody clusters (see Figure 1B), and analysis was performed using the v test (see Patients and Methods), followed by Fisher's 2-sided exact test for rarer outcomes. Values are the percentage, except for age at onset, which is the mean. RNAP III = RNA polymerase III; topo I = topoisomerase I; GAVE = gastric antral vascular ectasia.
† $P \leq 0.001$ versus the overall mean, as determined by v test.
‡ $P \leq 0.05$ versus the overall mean, as determined by v test.
§ $P \leq 0.01$ versus the overall mean, as determined by v test.
¶ Significant association confirmed by Fisher's 2-sided exact test.
# RNAP III strong versus RNAP III weak significantly different by Fisher's 2-sided exact test.

Table 4. Clinical characteristics of the rarer SSc-associated autoantibodies*

Antibody, clinical association	Negative	Positive	*P*†	Adjusted *P*‡
TRIM-21/Ro 52				
Limited disease	227/327 (69)	143/178 (80)	0.001	0.47
Raynaud's phenomenon	305/327 (93)	174/178 (98)	0.038	0.049
Telangiectasia	283/327 (87)	171/177 (97)	<0.001	0.001
Calcinosis	128/327 (39)	87/177 (49)	0.031	0.092
Dry eyes	208/327 (64)	131/178 (74)	0.023	0.051
PAH	31/327 (10)	30/178 (17)	0.016	0.073
CENP	126/327 (39)	102/178 (57)	<0.001	–
PM/Scl				
Limited disease	297/416 (71)	73/89 (82)	0.042	0.052
Digital ulcers	204/416 (49)	54/89 (61)	0.047	0.032
Smoking, ever§	211/416 (51)	27/89 (30)	<0.001	<0.001
CENP	178/416 (43)	50/89 (56)	0.021	–
Th/To				
Joint contractures§	231/490 (47)	2/15 (13)	0.015	–
Reflux esophagitis§	280/490 (57)	4/15 (27)	0.031	–
Ku				
Telangiectasia	444/491 (90)	10/14 (71)	0.041	–
Fibrillarin	3/491 (1)	3/14 (21)	<0.001	–
Fibrillarin				
GAVE	37/499 (7)	2/6 (33)	0.071	–
Digital amputation	66/499 (13)	3/6 (50)	0.036	–
Ku	11/499 (2)	3/6 (50)	<0.001	–
CENP§	228/499 (46)	0/6 (0)	0.035	–

* Values are the number with the feature/total number in the group (percentage). TRIM-21 = tripartite motif–containing protein 21; PAH = pulmonary arterial hypertension; GAVE = gastric antral vascular ectasia.
† Determined by v test function, except for Th/To, Ku, and fibrillarin, which were determined by Fisher's exact test.
‡ Where the number of observations permitted, the *P* values were adjusted for autoantibody cluster/group.
§ Reduced frequency in the presence of the autoantibody.

The possible difference between the 2 clusters lies with the risk of GAVE and its association with RNAP III strong and with esophageal dysmotility, for which RNAP III strong is less likely. There was a trend for shorter disease duration for cluster RNAP III strong as compared with cluster RNAP III weak (Supplementary Figure 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39316/abstract).

Other SSc-associated autoantibodies. A univariate analysis was performed to look for clinical associations with the presence of TRIM-21/Ro 52, NOR-90, PM/Scl-75, PM/Scl-100, Th/To, Ku, fibrillarin, and PDGFR. Statistically significant associations or those approaching statistical significance are summarized in Table 4. Given the low numbers of patients with Th/To (15 of 505), Ku (14 of 505), fibrillarin (6 of 505), NOR-90 (19 of 505), and PDGFR (2 of 505), we report any associations with caution, recognizing that larger numbers are required to provide definitive results. Taking these limitations into consideration, patients with Th/To were less likely to have joint contractures ($P = 0.015$) and reflux esophagitis ($P = 0.031$). Ku and fibrillarin frequently occurred together ($P < 0.001$), and the presence of fibrillarin was associated with digital amputation ($P = 0.036$) and a trend toward the presence of GAVE ($P = 0.071$). No significant associations were observed for NOR-90 and PDGFR.

TRIM-21/Ro 52 and PM/Scl-75 or PM/Scl-100 were the most common of the other SSc-associated autoantibodies, with 35.2% and 17.6% of patients testing positive for these autoantibodies, respectively. While their presence did not contribute to subclassification of the patients, the clinical associations for TRIM-21/Ro 52 and PM/Scl-75/100 are of interest and are summarized in Table 4. The presence of telangiectasia was strongly associated with TRIM-21/Ro 52 ($P < 0.001$), while adjusted data showed a trend toward association with the presence of dry eyes, PAH, and calcinosis. There was no evidence of an association with ILD in this cohort ($P = 0.30$).

PM/Scl-75/100 was associated with a history of digital ulcers (adjusted $P = 0.032$), and patients expressing this autoantibody showed a trend toward the presence of limited SSc (adjusted $P = 0.052$). PM/Scl-75/100 was more commonly identified in those who had no history of smoking, the reason for this is uncertain and may relate to as-yet-unidentified confounding factors. Both

TRIM-21/Ro 52 and PM/Scl-75/100 were more commonly seen in the presence of CENP.

DISCUSSION

A scoring system that identifies both the presence and intensity of autoantibody staining reduces bias from multiple autoantibody positivity and reduces confounding from false-positive results. The presence of autoantibodies does not change over time (24,25), although the staining intensity may vary. Other investigators have found that autoantibodies with the highest titer are the most strongly associated with the clinical phenotype (14) and may be more reliable than clinical skin scores, which are subject to interobserver variability (26,27). Our findings extend these observations by demonstrating that this remains true even when possible modulation of disease by the presence of multiple positive autoantibodies is considered.

In the presence of multiple autoantibodies, CENP, topo I, and RNAP III remained the most common SSc-specific autoantibodies in this cohort and had strong clinical associations. While coexpression of any of these 3 major autoantibodies remained rare (Table 2), coexpression with other autoantibodies has been found to be frequent in our cohort and others (13,14,21). Principal components analysis followed by hierarchical clustering provides a novel means by which to interpret these complex associations by considering only the dominant autoantibody, and it fosters uniformity within each autoantibody cluster.

Patients positive for cluster CENP (41%) were predominantly female and showed significant associations with calcinosis, reflux esophagitis, sicca symptoms, telangiectasia, anal incontinence, and PAH. In this cluster, ILD, joint contractures, tendon friction rubs, and synovitis were reduced as compared with the other clusters. The clinical associations of CENP remain across geographic locations and ethnic groups (24,28–30) and provide a universal prognostic marker.

RNAP III was associated with 2 different autoantibody clusters, depending on the intensity of RNAP III staining (cluster RNAP III strong [8%] and cluster RNAP III weak [5%]). RNAP III titers change over time (31), and in our analysis, there was a trend toward increased titers in patients with earlier disease. Therefore, clusters RNAP III strong and RNAP III weak may represent different temporal stages of SSc disease. The trend toward cluster RNAP III strong representing earlier disease and an association with scleroderma renal crisis and GAVE is consistent with other studies, where both scleroderma renal crisis and GAVE were found to be more likely to occur in early disease (32–34). Patients in cluster RNAP III strong appeared less likely to have esophageal dysmotility, which is an intriguing finding. Perhaps the invasive nature of assessing and confirming esophageal dysmotility may see it underreported in our cohort, as other studies have reported early involvement of the gastrointestinal tract even in the absence of symptoms (35,36).

Prevalence rates for RNAP III vary according to ethnicity and geographic location. The frequency of RNAP III in this cohort lies somewhere between the rates identified in other international cohorts (13,20,21,37–42). Cluster RNAP III strong patients had the highest MRSS, while cluster RNAP III weak patients had the second highest MRSS, equaling that for cluster topo I (Supplementary Figure 4, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39316/abstract). The clinical associations of high MRSS, scleroderma renal crisis, and now GAVE are consistent globally even though the prevalence rates for this autoantibody vary (39,43).

Previous studies have found an association of the diagnoses of SSc and malignancy with RNAP III (19,44,45), while our study did not. One possible reason for this finding is that in our study, malignancy was defined as either hematologic or solid tumor, with nonmelanoma skin cancers excluded. Nonmelanoma skin cancer is by far the most common cancer diagnosed in Australia (46).

The remainder of patients who had a clinical association with diffuse SSc were captured in cluster topo I (18%). Topo I frequencies were consistent with the frequencies reported in other international registries of similar ethnicity but varying geographic locations, and it should be noted that the frequencies of this autoantibody vary with ethnicity (13,14,20,21,28,30,37,38,40–42,47–49). Significant clinical associations in this cohort include high MRSS, ILD, joint contractures, and digital ulcers.

Coexpression between the 3 major autoantibodies was only rarely observed in this cohort (Table 2), but warrants further discussion. Their presence may represent a false-positive result, but if this is the case, they are a consistent finding in other published work (14,20). It would be intriguing to investigate this group further to determine whether they share any clinical features, particularly with relation to disease onset and severity. Given their rarity, meaningful analyses will likely require collaboration between national and international disease registries. Among other investigators whose published studies used this technology, Mierau et al (21) from the German Network for Systemic Scleroderma Registry used a commercial line immunoassay (Euroimmun) and found that coexpression of any combination of CENP, RNAP III, and topo I was rare, while coexpression of additional

nonspecific autoantibodies was common. In addition, Graf et al (14), who used the same assay as we did, reported the coexpression of both specific and nonspecific autoantibodies. They concluded that the clinical phenotype was most strongly represented by characteristics generally associated with the dominant autoantibody, and those findings were replicated in our study.

Cluster other (28%) captured the remaining patients in our cohort. This cluster included patients who 1) did not express CENP, topo I, or RNAP III, 2) may have had a low score for 1 of the traditional SSc autoantibodies coupled with 1 or more of the nontraditional autoantibodies, or 3) may have had either monospecificity or multispecificity for the nontraditional autoantibodies.

Positive associations in this cluster included male sex, a history of malignancy, and a history of smoking. Negative associations included telangiectasia, Raynaud's phenomenon, and joint contractures. The clinical associations in this group are intriguing and warrant further investigation. U1 RNP was not available on the SSc immunoblot; however, in separate testing of all patients for this autoantibody, all those classified as having MCTD mapped to cluster other (data not shown). If U1 RNP had been included on the immunoblot, it may have formed a separate cluster with its own specific clinical associations. It is likely that cluster other identifies more than 1 disease group.

The rarer SSc-associated autoantibodies were also assessed in this study, but they were not found by principal components analysis to significantly contribute to subclassification. Only a small number of patients tested positive for NOR-90, PDGFR, Ku, fibrillarin, and Th/To, and it is likely that large multinational studies will be necessary to determine if they have significant clinical associations.

Where statistical power permitted, both TRIM-21/Ro 52 and PM/Scl were found to have more meaningful results. TRIM-21/Ro 52 is a common autoantibody, and its presence in SSc has been reported elsewhere, with varying clinical associations (13,14,21,50). Of particular significance with regard to disease prognostication are the reports of associations with overlap disease and ILD (13). In this cohort, the presence of TRIM-21/Ro 52 was associated with telangiectasia and Raynaud's phenomenon, with a trend toward the presence of dry eyes, calcinosis, and limited disease. These clinical features are consistent with the findings of other investigators who have reported associations with limited disease (14,21) and sicca features (21). The trend toward PAH is intriguing and has not been reported elsewhere, although direct comparison between the groups is difficult as right-sided heart catheterization is not readily available in all cohorts. The Canadian Scleroderma Research Group registry (13) used a surrogate marker obtained from the echocardiogram (pulmonary arterial hypertension), while Mierau et al (21) did not provide a detailed definition of pulmonary hypertension. Differing methods used to detect TRIM-21/Ro 52 may also account for variations between cohorts. However, it is possible that the use of right-sided heart catheterization may improve the specificity of any findings in relation to PAH. This could be clarified by review in a larger group.

Unlike Hudson et al (13), we did not find an association between Ro 52 and ILD, although the definition for ILD varied significantly between the 2 studies. In our cohort, ILD was defined on the basis of radiologic findings, while in the Hudson et al cohort, a recently published algorithm for ILD, which included patients with typical clinical findings in the absence of radiologic findings, was used.

The presence of PM/Scl-75/100 in SSc has been associated with limited and overlap disease, skeletal muscle disease, calcinosis, improved survival (21,51,52), and more recently, improved prognosis in those with ILD (53). Techniques used to detect these autoantibodies varied considerably between studies, and this must be taken into account when interpreting the findings between the 2 cohorts. In our cohort, PM/Scl-75/100 was associated with digital ulcers, and there was a trend toward an increase in the presence of PM/Scl-75/100 in those with limited disease. No significant associations with a history of myositis or elevated creatine kinase were observed (Patterson KA, et al: unpublished observations).

SSc is traditionally classified according to the extent of skin fibrosis. Limited SSc and diffuse SSc have well-established differences in their presentation, autoantibodies, and outcomes. However, the dermatologic changes are a dynamic process, and hence, early identification of the SSc as being limited may need to be modified during the disease course.

The difficulties in the diagnosis and subclassification of SSc based on skin involvement have direct relevance in the clinical setting. Virendrakumar Bhavsar et al (54) recently described 2 cases of RNAP III–associated scleroderma renal crisis occurring in the absence of initial skin involvement, which presented a major diagnostic challenge. Furthermore, Cottrell et al (55) concluded that dichotomous classification based on skin involvement may result in the misclassification of an intermediate group of patients who have a unique autoantibody profile, disease course, and clinical outcome.

In contrast, autoantibodies are a consistent feature of the disease, and it is rare for an autoantibody to disappear, although fluctuations in antibody titer or binding

intensity may occur. Our analysis has shown significant clinical correlations within each cluster in the absence of any other information apart from the diagnosis of SSc and the presence and staining intensity of the autoantibody. It therefore seems logical to propose that disease stratification based on autoantibodies would be simpler to apply and would be more consistent, irrespective of disease stage, than the traditional classification system based on the extent of skin involvement. Furthermore, this cross-sectional study demonstrated strong disease associations with the autoantibodies and may have important applications in the enrichment of studies of new therapies for certain subsets and in the identification of patients for targeted therapies.

There were minimal missing data, and this allowed comprehensive statistical analysis of the results of the autoantibody testing in relation to the clinical and demographic variables. Limitations of the study include the small number of patients with rarer autoantibodies. While the clinical associations with the autoantibodies in this study were similar to those found in other published studies, it is important to recognize that there may be different associations depending on the way the autoantibodies are identified and according to the various platforms currently in use. Autoantibody identification by assays that target a specific autoantibody peptide sequence does not necessarily reflect the clinical associations that are recognized through identification by more labor-intensive assays that detect the whole protein or indeed through identification by functional assays. The sensitivity often reported with the use of line immunoassays may result in changes to previously reported disease associations, and therefore, it is recommended that the performance of the newer technologies be assessed in well-characterized SSc registries.

Principal components analysis provides a novel means by which to identify the presence and intensity of scleroderma-associated autoantibodies and to reduce confounding when more than 1 autoantibody is detected in patient sera. We have shown that subclassification based only on the presence of autoantibodies reveals clinically meaningful disease associations. The dominant autoantibody is the one that most accurately reflects disease associations, at least when CENP, topo I, and RNAP III are considered. Two separate clusters of RNAP III were identified, with intriguing differences in their clinical associations. We propose that this may relate to the well-documented fluctuations in RNAP III over time, with higher titers being observed earlier in the disease course. Additional prospective studies will provide further clarification in this regard. In our cohort, those who did not map to 1 of the 4 major autoantibody-associated clusters had intriguing clinical associations. They are likely to represent more than 1 disease group and warrant further investigation.

We conclude that the use of specific and associated SSc autoantibodies provides more meaningful classification of subsets than the currently used subdivisions of limited, diffuse, and overlap. We also propose that using autoantibody profiling for subclassification and stratification will improve disease management and prognostication as well as the identification of patients for entry into clinical trials.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Walker had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Roberts-Thomson, Walker.

Acquisition of data. Patterson, Roberts-Thomson, Rischmueller, Zochling, Sahhar, Nash, Roddy, Hill, Nikpour, Stevens, Proudman, Walker.

Analysis and interpretation of data. Patterson, Roberts-Thomson, Lester, Tan, Hakendorf, Walker.

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