THE UTILITY OF AUTOANTIBODIES AS BIOMARKERS IN A WELL CHARACTERISED AUSTRALIAN SYSTEMIC SCLEROSIS (SCLERODERMA) COHORT

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GLOSSARY

Acronym/abbreviation	Meaning
AA/s	autoantibody/autoantibodies
ACA	anti-centromere antibodies
ACE	angiotensin-converting enzyme
ACR	American College of Rheumatology
Ag	antigen
AIM	autoimmune myositis
ALBIA	addressable laser bead immunoassays
ANA	antinuclear antibody
ANOVA	analysis of variance
ASCS	Australian Scleroderma Cohort Study
ASIG	Australian Scleroderma Interest Group
Blot	immunoblot
C.I.	confidence interval
cDNA	complimentary deoxyribonucleic acid (DNA)
CENP	Centromere protein (A or B)
CIE	Counter immuno-electrophoresis
CK	creatine kinase
CLIA	chemiluminescent immunoassay
CTD	connective tissue disease
dcSSc	diffuse cutaneous systemic sclerosis
DID	double immunodiffusion
DLCO	diffusing capacity of the lungs for carbon monoxide
DM	dermatomyositis
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
ENA	extractable nuclear antigen
EULAR	European League Against Rheumatism
FEIA	fluoro-enzyme immunoassay
GAVE	gastric antral vascular ectasia
GWAS	genome wide association studies
He-La	HeLa cell line, originally derived from a tumour from Henrietta Lack, a cancer patient.
HE-p2 cells	human epithelial type 2 cells
HLA	human leukocyte antigen
HSCT	Autologous hematopoietic stem cell transplant
hUBF/NOR90	human upstream binding factor
IB	immunoblotting
IgG	Immunoglobulin G
IIF	indirect immunofluorescence
IIM	idiopathic inflammatory myopathies
ILD	Interstitial lung disease
IP	immunoprecipitation
IPF	idiopathic pulmonary fibrosis
kDa	Kilo Dalton
lcSSc	limited cutaneous systemic sclerosis
LIA	line immunoassay
LSSc	Localised systemic sclerosis/morphea

Acronym/abbreviation	Meaning
mRNA	messenger ribonucleic acid
MCTD	mixed connective tissue disease
mRSS	modified Rodnan Skin Score
NHC	normal healthy control
PAH	pulmonary arterial hypertension
PBC	primary biliary cirrhosis
PCA	principal component analysis
PDGF/R	platelet derived growth factor/receptor
PF	pulmonary fibrosis
PmScl	polymyositis/scleroderma
RA	rheumatoid arthritis
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
RNAP3	RNA Polymerase III
RNase MRP	RNA Mitochondrial RNA processing complex
RNase P	Ribonuclease P
ROS	reactive oxygen species
RP	Raynaud's Phenomenon
RR	relative risk
rRNA	ribosomal ribonucleic acid
SARD	systematic autoimmune rheumatic disease
SASR	South Australian Scleroderma Register
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SjS	Sjogren's syndrome
SLE	systemic lupus erythematosus
SMR	standardised mortality ratio
snoRNP	small nucleolar ribonucleoprotein
snRNP	small nuclear ribonucleoprotein
SRC	scleroderma renal crisis
SSc	systemic sclerosis
ssSSc	scleroderma sine scleroderma
TGF-β	transforming growth factor β
Topo1	topoisomerase 1/DNA topoisomerase 1
TRIM21/Ro52	tripartite motif-containing protein 21
tRNA	transfer ribonucleic acid
U1RNP	U1 ribonucleoprotein
USA/US	United States of America
WB	western blot

SUMMARY/ABSTRACT

Utility of Autoantibodies as Biomarkers in a Well Characterised Australian Systemic Sclerosis (Scleroderma) Cohort

Background

Systemic Sclerosis is a clinically heterogeneous systemic autoimmune disease of unknown aetiology. Autoantibodies (AAs) are present in >95% of patients. Three AAs were originally considered to be highly associated with SSc; Centromere protein (CENP A or CENP B), Topoisomerase1 (Topo1) and RNA Polymerase III (RNAP3) and all were closely linked with distinct clinical manifestations. Initially it was thought that AAs were mutually exclusive and patients expressed only a single AA, however more recent technologies have demonstrated that multiple AAs can be expressed in a single patient and that other serum AAs are associated with SSc. Some of these later AAs were only available in a research setting, with their clinical associations and frequencies obscure.

Further uncertainties regarding AA's in scleroderma include the relevance of multiple AA positivity, and that of AA negative SSc. Lastly, the 2013 ACR/EULAR classification criteria for SSc showed improved diagnostic validity, but did not encompass sub-classification nor provide prognostication. Improved biomarkers for SSc subsets are sorely needed.

Aim

To determine the relationships between SSc related autoantibodies including their clinical associations in a large and well-characterized Australian patient cohort using a single diagnostic platform to detect multiple AAs.

Hypothesis

Important relationships between AAs and their clinical associations will identify and stratify AAs into clinically homogeneous subgroups.

Method

The (Euroimmun) line immunoblot assay (LIA) was used to characterise antibodies to CENP-A, CENP-B, RNAP3; epitopes 11 and 155, Topo I, NOR-90, Fibrillarin, Th/To, PM/Scl-75, PM/Scl-100, Ku, TRIM21/Ro52, and PDGFR in 505 Australian SSc sera. Supplementary LIA testing of U1RNP was also performed in selected patients.

Statistical Analyses

Patient subgroups were identified by hierarchical clustering in a principal components analysis (PCA) of quantitative autoantibody scores. Results were compared with detailed clinical data.

Results

A total of 449/505 patients were positive for at least 1 AA by LIA. Heatmap visualization of AA scores, along with PCA clustering, demonstrated strong, mutually exclusive relationships between CENP, Topo I and RNAP3. Five patient clusters were identified: CENP, RNAP3 strong, RNAP3 weak, Topo I, and ‘Other’. Clinical features associated with CENP, RNAP3, and Topo I were consistent with previously published reports concerning lcSSc and dcSSc. A novel finding was the statistical separation of RNAP3 into two clusters. Patients in RNAP3 strong cluster had an increased risk of gastric antral vascular ectasia, but a lower risk of oesophageal dysmotility. Additional PCA of Cluster 4 revealed that Topo1 and CENP maintained their clinical influence even at reduced LIA staining intensity with co-expressed CENP/Topo1 patient phenotype resembling Topo1 with minimal CENP influence. A statistically significant presence of males in this and the AA negative subgroup. The AA negative subgroup phenotype was more fibrotic and less vasculopathic. Clinical associations for TRIM21 included older age at disease onset and a tendency towards ILD. SSc positive U1RNP patients and U1RNP MCTD were different, the latter having a milder phenotype.

Conclusion

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

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Karen Patterson

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'I can't change the direction of the wind…but I can adjust my sails to always reach my destination…' *Jimmy Dean*

CHAPTER 1
LITERATURE REVIEW; THE UTILITY OF AUTOANTIBODIES AS BIOMARKERS IN SYSTEMIC SCLEROSIS, (SCLERODERMA).

Systemic sclerosis (SSc) is a heterogeneous autoimmune rheumatic disorder bearing the hallmarks of fibrosis of the skin and visceral organs, a widespread micro-vasculopathy and dysregulation of the innate and adaptive immune systems (1). Despite rigorous examination, no one unifying aetiology or pathogenesis has been found. The likely contributors to developing SSc include genetic susceptibility (2-5) and environmental interaction (6-8) with unknown and/or stochastic factors (including the effects of epigenetics) (9-11), playing a pivotal role.

This review begins by summarising the clinical course and disease outcomes of systemic sclerosis. Strategies to improve disease outcome are discussed with a focus on recent advances in disease classification as a means to increase early detection of disease. There follows a detailed discussion of scleroderma related autoantibodies and their clinical associations. It concludes with hypotheses to investigate the role of an extended panel of disease-associated autoantibodies (AAs) in disease sub classification and prognostication.

Clinical Course

Systemic sclerosis is a rare disorder, affecting approximately 23 individuals per 10^5 (10) of the Australian population. Like most autoimmune diseases it occurs more frequently in women and presents most commonly in the 4^{th}- 5^{th} decade of life (12). The majority of patients present with a history of Raynaud's phenomenon, skin changes, characteristic AA findings and nailfold capillaroscopy changes, in addition to a varying degree of major organ involvement (13).

Generally, two major clinical presentations (limited and diffuse SSc) are recognised, and these have been characterised according to the degree of skin involvement (Figure 1-1). These broad subtypes have been linked with classic AA expression. An archetypal patient with limited cutaneous SSc (lcSSc) would present with a history of Raynaud's phenomenon, predating their skin changes by some years. Sclerodactyly is limited to the peripheries, and frequently accompanied by telangiectasia, calcinosis and oesophagitis. In a small percentage, pulmonary arterial hypertension (PAH) may occur, usually some years after the onset of disease (13).

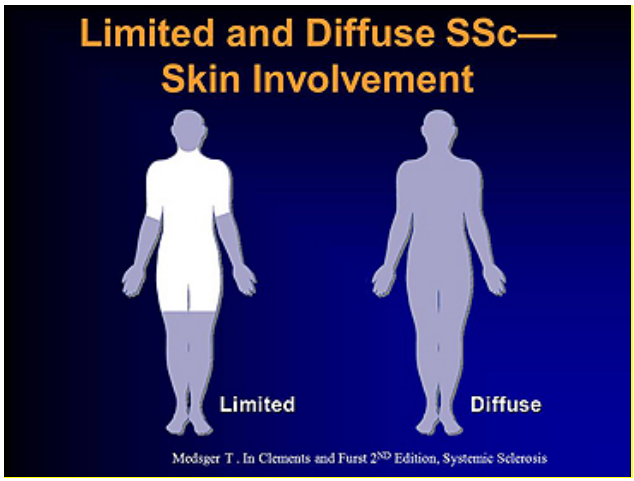


Figure 1-1: Skin involvement in systemic sclerosis, limited vs diffuse disease.

In contrast, patients with diffuse cutaneous SSc (dcSSc) present with rapid progressive skin change involving the chest wall and proximal limbs as well as the peripheries. Raynaud's phenomenon occurs simultaneously with the onset of skin fibrosis, or precedes fibrosis by only a short time period. Patients with diffuse disease are more likely to develop the feared complications of pulmonary fibrosis or renal crisis. Occasionally patients are also seen with localised scleroderma (LSSc or morphea) and scleroderma sine scleroderma (ssSSc) where typical AA and other skin changes are identified in the former but the skin is spared in the latter (Table 1-1). Figures 1-2 to 1-8 show the clinical manifestation of various common symptoms in dcSSc and lcSSc (14) .

Table 1-1: Clinical summary of the four major SSc sub types

Subtype	Clinical presentation
Diffuse cutaneous SSc (13) (dcSSc)	Raynaud's phenomenon onset simultaneously or within 1 year of skin changes Proximal skin fibrosis up to elbows and knees including trunk Rapidly progressing skin fibrosis Nailfold capillary dilatation and destruction Characteristic AAs (Anti topoisomerase I, RNA Polymerase III) Interstitial lung disease, renal complications (renal crisis), diffuse gastrointestinal disease, myocardial involvement Tendon friction rubs may be present
Limited cutaneous SSc (13) (lcSSc)	Raynaud's phenomenon onset for years before skin involvement Skin involvement limited to hands, face, feet Nailfold capillary dilatation with less destruction Significant (10-12%) late onset PAH Gastrointestinal complications Sclerodactyly, telangiectasia and calcinosis
SSc Sine Scleroderma (13)	No detectable skin involvement Raynaud's phenomenon Nailfold capillary abnormalities PAH
Localised scleroderma (LSSc) (Morphea) (15)	Five subtypes; plaque, localised, linear, bullous and deep. No serious systemic manifestations (with exceptions) Neurological and ocular manifestations (possible) Fibrosis limited to skin and subcutaneous tissues

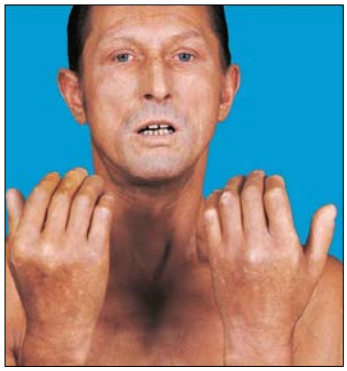


Figure 1-2: dcSSc presentation, sclerotic skin, microstomia (14)

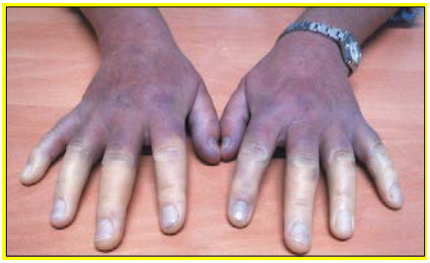


Figure 1-3 Raynaud's Phenomenon (14)

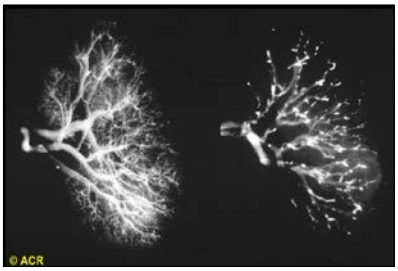


Figure 1-5 Vasculature, normal (left) vs SSc (right) kidney. Source: ACR

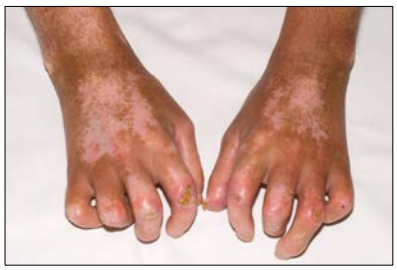


Figure 1-4 Sclerodactyly, skin pigmentation and digital ulcers (14)

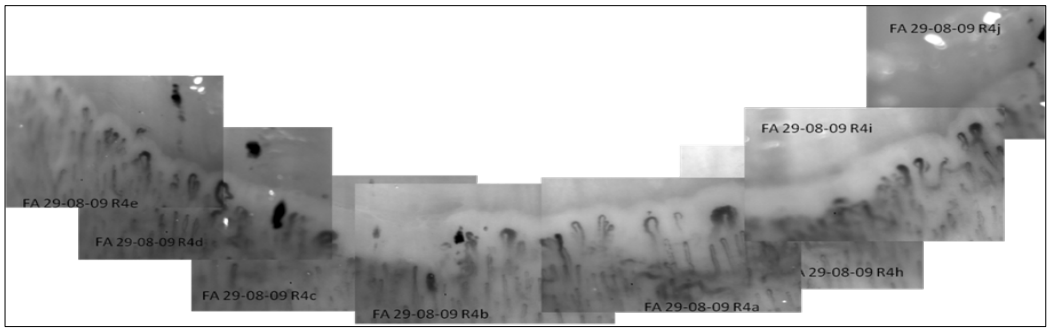
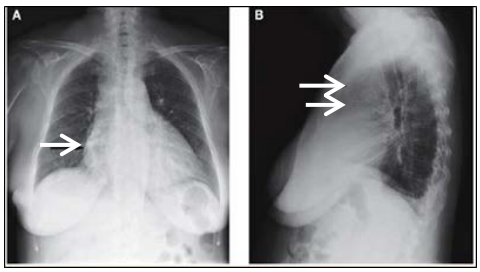


Figure 1-6: Diffuse nailfold capillaries; dilatation, dropout, leakage, disordered appearance. Image: K.A. Patterson



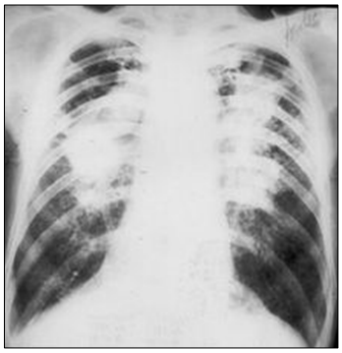


Figure 1-8 ILD in SSc.

Figure 1-7: End stage PAH. Arrows indicate dilatation right atrium (A) and right ventricle (B).

Disease Outcomes - Prognosis

Among the systemic autoimmune rheumatic diseases (SARD), SSc has the worst outcome with dcSSc having the highest standardised mortality ratio (SMR) of all systemic rheumatic disorders of 4.73 (95%CI 3.69–6.07) (16, 17). In addition SSc also has a profound effect on quality of life, although the societal, emotional and economic costs to sufferers and their families are difficult to accurately determine because of the multifactorial comorbidities (18). The predominant cause of death has changed in the past few decades from renal crisis to the pulmonary complications of interstitial lung disease (ILD) and PAH (17).

In a meta-analysis of mortality and survival in SSc by Rubio-Rivas et al (17) it was reported that 47.6% of all deaths were SSc related with 73% of those deaths attributed to cardiopulmonary involvement. They also reported that renal and gastrointestinal related deaths had fallen over the past 20 years with these complications now representing 18% of SSc related deaths. They have attributed the decline in deaths from renal complications to the introduction of angiotensin converting enzyme (ACE) inhibitors. They estimated that dcSSc patients had a SMR of 4.73 (95%CI 3.69–6.07) and lcSSc SMR was estimated at 2.04 (95%CI 1.55–2.68). There were also differences between genders with the male SMR estimated at 3.14 (95%CI 2.62–3.76) and females at 2.93 (95%CI 2.36–3.64).

The Pittsburgh Scleroderma Databank (n=1432) compared patient survival by disease classification as well as AA subset and found that patient survival is closely related to both the subset, (limited or diffuse), and the antibody present (19). In this study, AAs associated with lcSSc or dcSSc classifications were investigated within their disease classification subsets to control for bias in disease duration. Table 1-2 shows the results from this study in which Steen states that most lcSSc patients have either anti-centromere antibodies (ACA), Th/To, U1RNP or PmScl AAs. In contrast, dcSSc patients have Topoisomerase 1 (Topo1), RNA Polymerase III (RNAP3) and fibrillarin AAs. Others have also explored the relationship between survival, disease subset and antinuclear antibody (ANA) status. A Japanese study of 275 patients by Kuwana et al (20) (Table 1-2) compared survival between both disease classification and AA subgroups. They reported '…clear differences in survival rates among the ANA based patient groups...it is reasonable to state that each ANA was associated with prognosis because of the associations of the ANA with the fatal complications. These data suggest that the survival rates are associated more strongly with serum ANA than with the disease classification…' A separate study by Hashimoto et al (21) (n=405) found that dcSSc and male gender were associated with a poor prognosis while ACA patients had a better prognosis.

In an Australian study by Graf et al (22), survival from the first symptom onset was examined in 285/331 patients in a deceased cohort where age at disease onset, classification and AA status were available. Patients with Topo1, RNAP3 and U1RNP were associated with significantly reduced survival compared to ACA. The authors noted that most deceased U1RNP were young women with mixed connective tissue disease (MCTD). Th/To, fibrillarin and Ku were infrequently found and any association with survival did not reach statistical significance. The conclusion from this study was that SSc specific AAs are associated with clinical phenotype and survival. Another study by Hissaria et al (23) found that diffuse skin involvement and male gender along with Topo1 and U1RNP were associated with a poor prognosis.

The Belgian Systemic Sclerosis Cohort study (24) (n=438) investigated 5 year survival of patients by skin classification and found that 39 (9%) of their patients had died. This high figure is perhaps due to patients with long standing disease being included in this study as it was made up of consecutive patients with SSc who were examined at Belgian teaching hospitals between 2006 and 2011. This study compared survival between LSSc, lcSSc and dcSSc. Time to death by Kaplan-Meier analysis was shorter for dcSSc patients compared to lcSSc and LSSc patients. Of those that died, 3, 9 and 10 patients had RNAP3, ACA or Topo1 AAs respectively with the remaining patient's AA positivity not stated.

Lastly, Nihtyanova and Denton (25) (n=234) compared AA subgroups with survival and found that patients positive for ACA, U1RNP and RNAP3 had a favourable outcome, while patients' positive for Topo1, Th/To and fibrillarin predicted a worse outcome. The finding of increased survival for RNAP3 in this cohort contrasts findings in other international cohorts. The authors attribute the improved survival to aggressive treatment with angiotensin-converting enzyme (ACE) inhibitors, prior to which there was an extremely poor survival. Table 1-2 also demonstrates survival in this cohort.

Table1-2: Survival in SSc, a comparison of international cohorts, AA status and disease (skin) classification. N/A= Data not available.

Autoantibody /Skin subset	10 year survival (%) Pittsburgh(19)	10 year survival (%) Japan(20)	15 year survival (%) United Kingdom (25)
lcSSc	N/A	83	N/A
U1RNP	88	72	78
Centromere	76	93	78
PmScl	72	Not found Japanese pts	N/A
Th/To	65	N/A	N/A
dcSSc	N/A	71	N/A
RNA Polymerase III	75	30	93
Topo1	64	62	57
Fibrillarin (U3RNP)	61	N/A	N/A

Overall, autoantibodies associated with a poor prognosis are Topo1 and RNAP3 with ACA, Pm/Scl and Ku having a more favourable prognosis (25). Survival outcomes are varied for U1RNP, Th/To and fibrillarin. The difference in survival outcome for the latter three AAs in various geographic locations may lie in genetic background, and indeterminate environmental influences.

The extent of skin thickening or fibrosis associated with diffuse disease also has a major influence on survival outcomes. Some studies have explored differences both within and between the major AA groups (ACA and Topo1) for fibrotic involvement (26), however it is clear that further studies are warranted to investigate the subtleties within AA subgroups for underlying fibrotic mechanisms to improve not only quality of life, but survival.

Causes of Mortality

Pulmonary Arterial Hypertension

Pulmonary arterial hypertension is prevalent in ~10% of SSc patients (27) and represents ~30% of deaths (28). It is reported to have a worse outcome than either idiopathic PAH or other connective tissue disease (CTD) related PAH with a recent meta-analysis concluding that the three year survival rate for SSc-PAH was only 56% (95%CI 51-61) (27).The reasons for the difference in PAH mortality/survival between the CTDs are unknown.

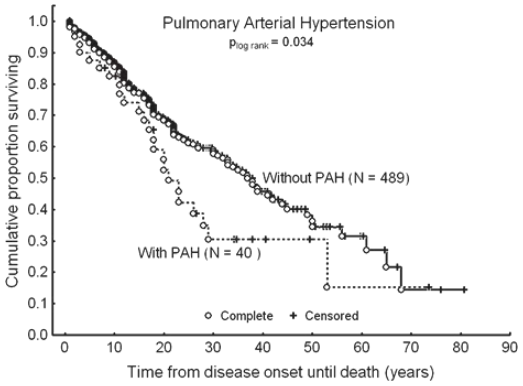


Figure 1-9 Kaplan-Meier cumulative survival curve of Scleroderma patients in the South Australian Scleroderma Register with pulmonary arterial hypertension. (Reproduced from (23))

Interstitial Lung Disease/Pulmonary Fibrosis

The Pittsburgh Scleroderma Databank evaluated patients over a 30 year period from 1972 to 2002 and found that pulmonary fibrosis as a cause of death increased from 6% to 33% over that time (29). The Canadian Scleroderma Research Group registry found the presence of ILD significantly contributed to mortality (p=0.006) in patients seen over a 10 year time period (30) while another Canadian study estimated the prevalence of SSc-ILD at 52% (31). In the South Australian Scleroderma Register (SASR) patients with ILD also had increased mortality with an adjusted relative risk (RR) of 2.34, with ILD present in 16% of dcSSc, 4% of lcSSc and 5% of overlap patients, (Figure 1-10) (23).

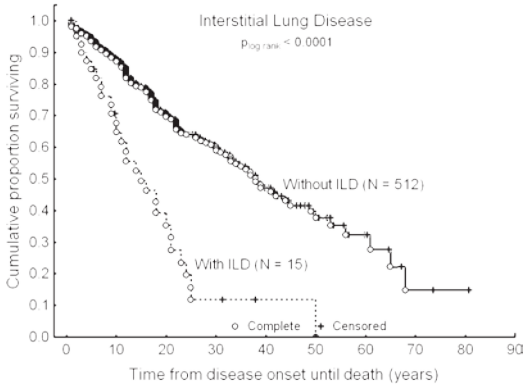


Figure 1-10: Kaplan-Meier cumulative survival curve of SSc patients in the SASR with ILD (23)

Scleroderma Renal Crisis (SRC)

Mortality from SRC has declined since the 1970's, presumably due to the advent of ACE inhibitors (32-36). Renal crisis now occurs in 4% - 10% of SSc patients, a great reduction considering historically it occurred in 25% of patients (35, 37) with mortality at 76% (38). In the SASR, 19 patients (4%) had SRC. These patients had the worst overall survival with almost half of these patients dying within 8 years of the disease onset, (Figure 1-11). Steen et al estimated SRC patient survival at five years between 50% to 70% (37).

Scleroderma renal crisis remains a severe complication of SSc and although survival has improved greatly, patients experiencing this complication still have a poor outcome with many on temporary or continuing dialysis (32, 35, 37).

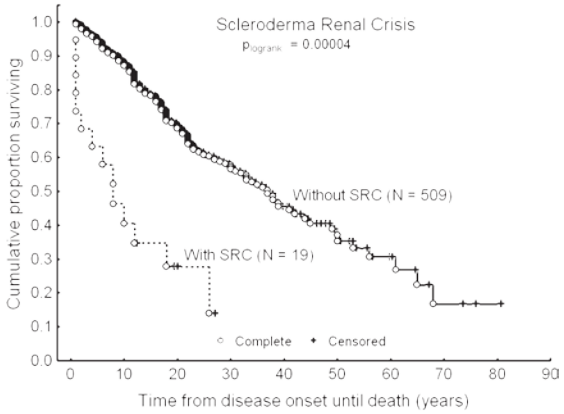


Figure 1-11: Kaplan-Meier cumulative survival of Scleroderma patients in the SASR with scleroderma renal crisis. (Reproduced from (23))

Strategies to improve outcome in Systemic Sclerosis

Treatment options in systemic sclerosis remain limited and are generally targeted towards specific organ involvement. Many of these are associated with significant toxicity with limited evidence of substantial benefit. ACE inhibitors are arguably the most successful treatment intervention for those who experience renal crisis but at this time they have not been shown to have benefit in disease prevention.

Autologous hematopoietic stem cell transplant (HSCT) therapy aims to provide a global reduction in disease activity but is also associated with significant morbidity and mortality. It was first used in SSc in 1996 (39) and 442 European SSc patients have had HSCT up until March 2016 (40). Although HSCT has shown some promise, of all autoimmune disease patients that had HSCT, SSc has the worst 5 year survival rate at 76% (95%C.I. 69-83%). The progression free survival rate is 55% (95% C.I. 69-83%) (41) and the 100 day transplant-related mortality rate is 10% (40). No currently available therapies have been shown to reverse fibrotic damage.

Significant improvements in disease outcome are likely to rely on accurate, early disease detection before irreversible damage has occurred. Early diagnosis and prognostication of patients has the potential to improve outcome by identifying those patients at greater risk of complications and allowing the institution of treatments in a timely fashion, either as standard therapy, or in the setting of a clinical trial.

Updating Diagnostic Criteria, and Classification in SSc

Early diagnosis of patients with SSc is critical to allow timely intervention of therapies, both in the setting of clinical trials and during routine management. In 2013, the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) published updated classification criteria for SSc (42) as the 1980 ACR classification lacked sensitivity and missed patients with early disease or with limited skin involvement (43). Underpinning the need for the reclassification was the recognised heterogeneity of SSc. The new classification criteria as described by Van den Hoogen et al, are:

…*intended to be used by rheumatologists, researchers, national and international drug agencies, pharmaceutical companies, or any others involved in studies of SSc. Our objective was to develop a set of criteria that would enable identification of individuals with SSc for inclusion in clinical studies, being more sensitive and specific than previous criteria* (43).

Ideally there should be no difference between classification criteria and diagnostic criteria, but in reality diagnostic criteria will tend to have a higher sensitivity and lower specificity, whereas classification criteria will maximize specificity. Three major AAs (ACA, anti Topo1 and anti RNAP3) are recognised to be almost exclusively associated with SSc and have been included in the ACR/EULAR revised criteria (43), but allowance is also made for the small number of patients (5-10%) who have SSc in the absence these autoantibodies (44) (Table1-3).

Table 1-3 The American College of Rheumatology/European League Against Rheumatism criteria for the classification of systemic sclerosis* (reproduced from (43))
* These criteria are applicable to any patient considered for inclusion in a systemic sclerosis 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 fibrosis, generalised morphea, eosinophilic fasciitis, scleroderma diabeticorum, scleromyxedema, erythromyalgia, porphyria, lichen sclerosis graft-versus-host disease, diabetic cheiroarthropathy).

Item	Sub- Item(s)	Weight/Score†
Skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints (sufficient criterion)	-	9
	Puffy fingers	2
Skin thickening of the fingers (only count higher score)	Sclerodactyly of the fingers (distal to the metacarpophalangeal joints but proximal to the proximal interphalangeal joints)	4
Fingertip lesions (only count the higher score)	Digital tip ulcers	2
	Fingertip pitting scars	3
Telangiectasia	-	2
Abnormal nailfold capillaries	-	2
PAH or ILD (maximum score is 2)	PAH	
	ILD	2
Raynaud's Phenomenon	-	3
SSc related AA (Topo1, CENP, RNAP3) (maximum score is 3)	Anti - Topo 1	
	Anti - CENP	
	Anti - RNAP3	3

† 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 systemic sclerosis.

It is hoped that these updated classification criteria will allow a greater and more accurate capture of those patients who have systemic sclerosis but they are not designed to provide any form of sub-classification or stratification.

The Role of Sub-classification in SSc

SSc is a heterogeneous disease and the differing clinical presentations, AA associations and genetics have led some to propose that it encompasses more than one condition (45). Therefore, accurate stratification of the disease is critical to identify and separate patients into groups that contain a similar clinical course and prognostic outlook.

In 1988 LeRoy et al proposed a method of capturing the heterogeneity within the disease (42) and in 2001 they amended the criteria so that patients with early SSc were recognised. Included in this revised classification criteria were autoantibodies and nailfold capillaroscopy (46) (Table 1-4). In the LeRoy and Medsger criteria (2001) (46), limited involvement describes skin involvement distal to the elbow and knees and above the clavicle, while those with diffuse disease have involvement both distally and proximal to these regions. This system is currently the most widely utilised to stratify patients.

Table 1-4. LeRoy and Medsger 2001 SSc

Category	Features
Limited SSc	Raynaud's Phenomenon (objective documentation) plus any one: SSc-type nailfold capillary pattern or SSc selective autoantibodies OR Raynaud's phenomenon (subjective) plus both: SSc-type nailfold capillary pattern and SSc selective antibodies
Limited Cutaneous SSc	Criteria for limited SSc plus distal cutaneous changes
Diffuse Cutaneous SSc	Criteria for limited SSc plus proximal cutaneous changes
Diffuse fasciitis with eosinophilia	Proximal cutaneous changes without criteria for limited SSc or limited cutaneous SSc

However there is recognition that these subsets are an over simplification of SSc (47-51) and others have proposed further modifications (45, 52-54), so that a precision medicine approach can be pursued (55-59). Given the considerable variation in SSc pathologies and the desire to improve treatments and interventions, stratification is vital when comparing outcomes and when designing clinical trials. The challenge now is to identify SSc patients that are more likely to develop severe organ involvement and also to determine the appropriate time to intervene before irreversible fibrotic or vascular damage occurs.

At present it can be difficult to stratify patients and compare research outcomes across varying geographic locations and ethnic (genetic) backgrounds merely using the broad lcSSc and dcSSc subsets. In addition to AA profiling to enhance patient stratification (56), utilising a precision medicine approach linked with HLA and non-HLA susceptibility genes identified in genome-wide association studies (GWAS) may lead to a better understanding of inheritance patterns, susceptibility, various pathogenic pathways and therapeutic targets (3, 60-62).

Finally, the interpretation and comparison of research outcomes and the stratification of patients would be enhanced if clinical associations were more closely aligned with focused subsets. One recent finding by Srivastava et al found that stratifying patients by skin involvement as well as AAs may predict clinical outcomes better than skin or serology alone in SSc (26). These findings can inform ongoing efforts to define more robust SSc subsets and perhaps it may become recognisable that skin in AA subsets may be due to biological variation within the AA subset rather than a function of an arbitrary title of lcSSc or dcSSc.

Biomarkers and SSc

'Biological markers' or 'biomarkers' are defined by the National Institute of Health Working Group on Biomarkers as a 'characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathological processes or pharmacologic responses to a therapeutic intervention' (63).

Biomarkers should be readily obtainable, quantifiable, objective, reproducible and able to act as a surrogate clinical endpoint with predictive power in different populations remaining at a similarly high degree of specificity and/or sensitivity. They should be available broadly and not only in an isolated research capacity. It is vital that there is an understanding between an assessable biological process and the clinical outcome they represent because biomarker outcomes may be the principal measure in drug development and other biomedical research enterprises (64). The utility of biomarkers includes prevention, diagnosis and early detection so that appropriate treatments can be developed and implemented before irreversible damage to the patients' health and wellbeing occurs. Further utility includes response to treatment, progression or cessation of disease.

Scleroderma, as with most autoimmune conditions, has a suite of biomarkers associated with various pathologies within the condition (51). Serum autoantibodies, found in >95% (65) of patients, are correlated with distinct clinical manifestations and have the potential, particularly in early disease, to aid in predicting disease course. There is potential to utilise AAs in conjunction with other biomarkers to predict fibrotic, vascular and organ manifestations and response to treatment. For example, a panel of SSc associated AAs could be interpreted in combination with information obtained from transcriptomics, proteomics, metabolomics, genomics and epigenomics to provide a detailed and individually personalised assessment of disease course (13). Theoretically this will be highly beneficial for patient outcomes as biomarkers based on precision medicine will refine treatments and therapies for which the patient is most likely to respond (66).

At present, sub classification for SSc is based upon a dichotomised skin based system (42) which is applied across a continuous spectrum of disease (45, 53). It is recognised that the current system means that important features of the disease process including serologic biomarkers and other organ involvement will be missed (53, 67). Furthermore, the skin changes that allow identification of disease subtypes may take some time to evolve and therefore have limited use in guiding therapy. Identification of a biomarker that can be reliably assessed before damage occurs, to both predict clinical outcomes and to determine appropriate intervention, has been arduous. To date, the most universal biomarkers remain the SSc associated

autoantibodies, found in 95% people with SSc (65). The following section of this review will describe the most relevant knowledge to date on autoantibodies as biomarkers in SSc including their association with clinical phenotypes and their utility in the sub classification of this perplexing condition.

Autoantibodies

Scleroderma associated autoantibodies are widely recognised to have distinct clinical associations but the validity of these findings will vary according to the diagnostic platform used. In addition, findings will be influenced by environmental and genetic factors (68).

Diagnostic Platforms

In recent years, numerous diagnostic platforms have been released to the commercial market, several without sufficient validation compared to conventional and standard methods (69). Because AAs recognise a variety of epitopes, it is vital to validate each test's sensitivity and specificity. Mahler et al explains (70):

…The diagnostic sensitivity is a statistical measure of how accurately a test correctly identifies diseased individuals...the diagnostic specificity is a statistical measure of how well a test correctly identifies absence of the disease in question…

It is also important to establish cut-offs for each assay that are based on the results from a range of local patients with SSc, other SARDs and healthy controls and that the cut-offs have been validated in other cohorts with differing demographic, geographic, environmental and genetic factors (71).

Indirect Immunofluorescence (IIF) on HEp-2 cells.

Screening for anti-cellular antibodies in subjects with suspected autoimmune disease using indirect immunofluorescence on HEp-2 cells test is one of the most common screening tests. The pattern obtained can provide information to assist in the diagnosis and classification of SSc and some other autoimmune diseases (72) with the exception of autoimmune myopathies. IIF is considered by some to be the 'gold standard' of anti-cellular antibody detection (73). The advantages of IIF on HE-p2 cells are that it is able to detect more than 100 different antigens including some that can be identified without a further confirmatory test (centromere). The disadvantages are that it is dependent on the experience of the reader, although there are automated systems available that can somewhat limit inter observer variation (74). It also has a low sensitivity for certain clinically important AAs (i.e., Jo-1 and other synthetase autoantigens, ribosomal P, SS-A/Ro60, Ro52/TRIM21) and, depending on the screening serum dilution, a low specificity (high false positive rate) (70). Further limitations include the nomenclature of patterns for nuclear (true ANA), cytoplasmic and mitotic staining, alternative platforms with different antigen profiles, standardisation, automation and incongruent results (75).

A negative IIF test does not exclude the presence of all connective tissue disease associated AAs and so where clinical suspicion is high, further testing should be

undertaken even in the presence of a negative IIF result (76) (77, 78).

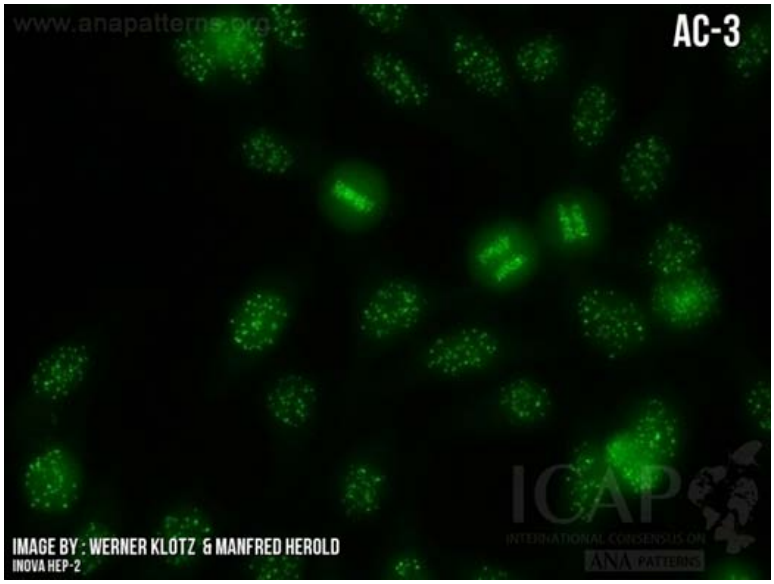


Figure 1-12: IIF Centromere pattern. Image ' ANA PATTERNS'. www.anapatterns.org

The Line Immunoassay (LIA)

The commercially available, qualitative LIA used in this study used 12 recombinant antigens that were expressed in one of the following vectors; Escherichia coli, insect or mammalian cells and in addition, a native antigen (Topo1) that was purified and isolated from calf and rabbit thymus. The advantages of this system are that it is relatively fast and allows for multiple AA detection of various staining intensity. Limitations regarding immunoblot results include a degree of protein denaturing. While some refolding may occur, conformational epitopes may be missed while previously hidden epitopes may now be exposed. For a description of the assay see Chapter 2, Methods, p.67.

Figure 1-13: Immunoblot work station

https://www.euroimmun.com/produkte/produkte-geraete-software/automatisierung-immunblot/euroblotone.html

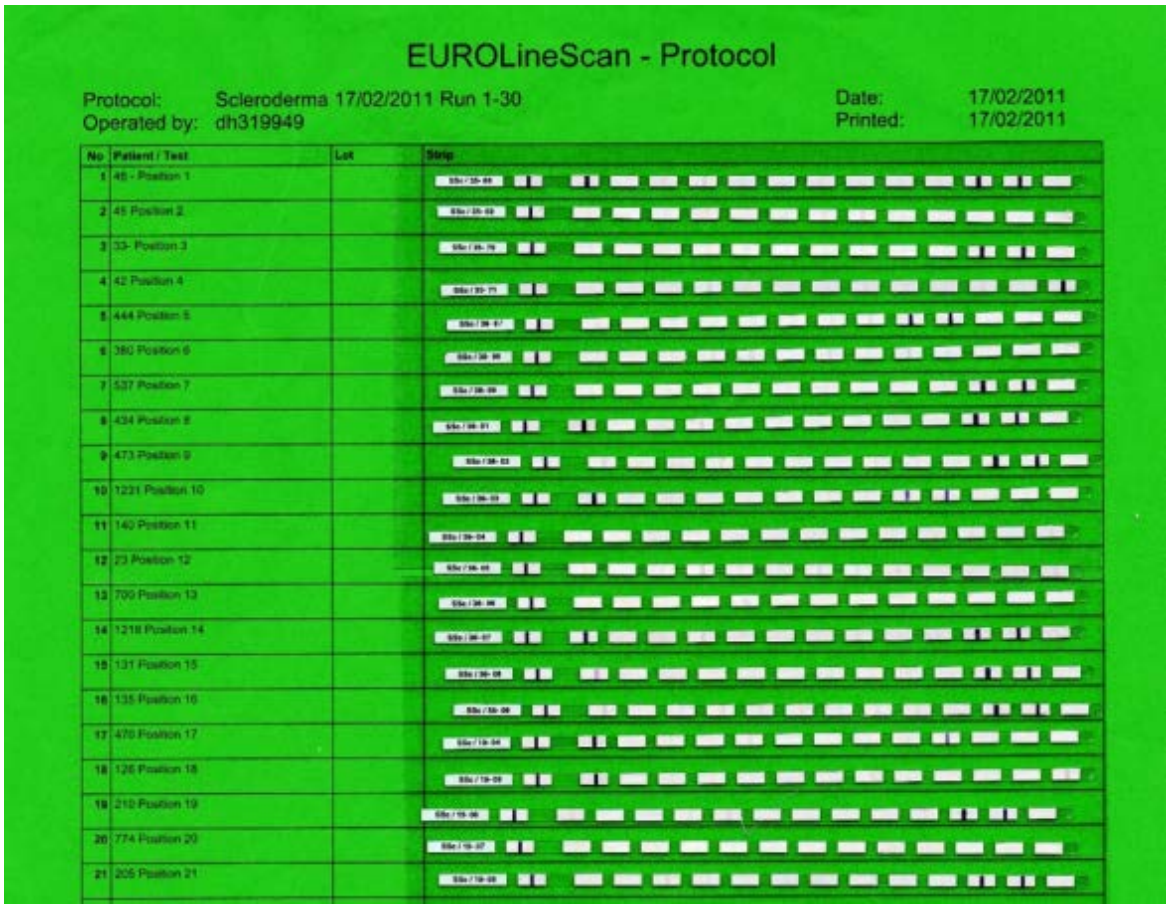


Figure 1-14: results - line blot assay Original Image: Karen Patterson

Enzyme linked immunosorbent assay (ELISA)

The original ELISA was a plate-based assay ('sandwich') technique designed for detecting and quantifying substances such as antibodies. It is routinely used in diagnostic laboratories with a variety of AAs available for testing in suspected autoimmune disease patients. There are also multiplex ELISA formats that adopt chemiluminescent/fluorescent reporter systems that use micro-bead based suspensions. The various ELISA formats (Figure 1-15), have been found to be reliable methods of AA detection (73, 79, 80). During the period of this study when sera were tested by the Australian Scleroderma Interest Group independent laboratories, the ELISA was the most utilised method for detecting RNA Polymerase III.

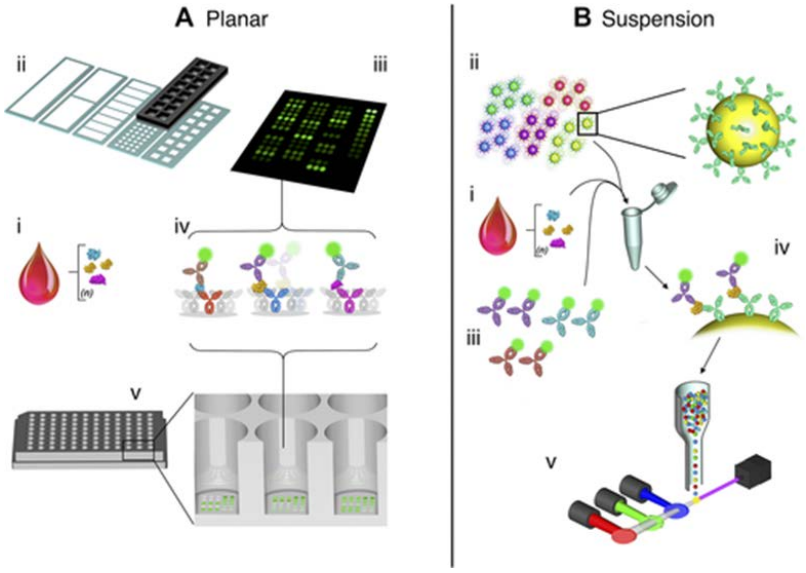


Figure 1-15 ELISA formats. The following is taken from Tighe et al (2015): (80)

Multiplex formats in common use include planar-based assays or suspension-based assays. (A) Planar arrays can be produced in two formats, either slide based or microtitre based. A common starting point for such assays, as with ELISA, would be a serum sample extracted from blood (i). Unlike microtitre plate based formats, slide-based formats support numerous layouts whereby repeated or individual assays composed of specific sets of antibodies are printed robotically upon the activated slide surface (ii). The sample matrix is applied and discrete assays are spatially separated by a frame and gasket, whereby they may be subsequently treated as individual microtitre wells, subject to blocking, washing, etc. Internal standards and replicates may be included also (C). Detection results from application of a composite of specific secondary antibodies coupled to a chemiluminescent/fluorescent reporter system (iv). Microtitre based immunoassays harbour regularly printed antibody sets within the confines of the wells of a standard (SBS format) protein-binding plate (v). The plate may thus be treated akin to a conventional ELISA (i.e. blocking, incubation and washing followed by detection with a set of reporter-conjugated detection antibodies). (B) Suspension immunoassays also have a common starting point – serum sample extracted from blood (i). This assay employs thousands of micrometre-sized plastic microbeads infused with a single (or several) chemiluminescent/fluorescent dyes and a functionally activated surface, prior to linking with a specific capture antibody. Numerous sets of such beads are prepared, each maintaining separate capture antibodies according to the cognate analyte and a unique fluorescent signature enabling identification (ii). The sample and a cocktail of all the requisite bead sets are thereafter combined. Sets of detection antibodies, all of which are individually labelled with a single chemiluminescent/fluorescent reporter (separate from those contained within the beads) are added upon completion of incubation and washing stages (iii). Each bead thus accommodates a ‘sandwich’ consisting of the captured target analyte and the cognate reporter-conjugated detection antibody (iv). Post-additional washing stages, bead analyte reporter constructs are subject to analysis in a flow chamber implementing individual bead separation, whereby lasers excite the chemiluminescent/fluorescent reporters and emitted light is collected by a series of detectors for quantitative analysis (v).

Extractable nuclear Antigen (ENA)/Immunoprecipitation (IP)

The simplest form of IP isolates a single target antigen to investigate the identity, structure, expression or activation of a protein that is immobilised on a solid support such as magnetic beads or agarose gel. It is one of the most widely used methods of protein isolation from cell or tissue lysates for the purpose of detection by other assay techniques and was commonly used by ASIG independent laboratories particularly for the AAs in Figure 1-16.

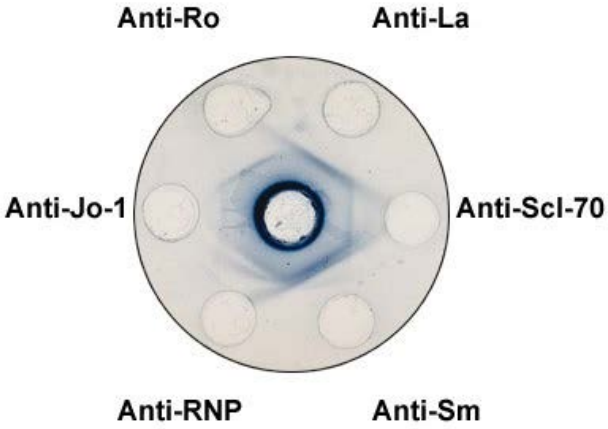


Figure 1-16: ENA/IP extract of rabbit or bovine tissue extracts that form precipitins with autoantibodies http://www.immunovision.com/ena-1001/

The source and characteristics of the autoantigen used in the various platforms must be considered as results may vary between recombinant, native peptide and full-length antigens. In particular, short polypeptide epitopes may give different results to an assay that detects reactivity to native antigens such as those represented in IIF cell based assays, immunoblotting or immunoprecipitation (76). In addition there are inter-manufacturer variations in reagents, in the standardisation of antigen (substrate) and the fixation process, nor is it a reliable method to detect antibodies to nucleic acids. Finally, there are the inter-laboratory variations in methods used, expertise, equipment and subjectivity in interpreting results.

Varying advantages and limitations apply to all platforms and it is most important to be aware of these considerations. Other techniques used to detect AAs include chemiluminescent immunoassays (CLIA), addressable laser bead immunoassays (ALBIA) and other microbead based assays, and nanobarcode arrays on planar surfaces (71). The latter are not widely available in diagnostic laboratories in Australia (70).

In short, no test is without its challenges and these should be considered when interpreting any AA result.

Primary SSc specific and SSc associated autoantibodies

There are three primary autoantibodies that are conventionally associated with SSc and considered to be highly specific for the disease; Anti Centromere antibodies (CENP), anti-Topoisomerase 1 (Topo1, formerly known as Scl-70) and anti-RNA Polymerase III. Earlier research hypothesised that these three autoantibodies were mutually exclusive and expressed in isolation, however with evolving diagnostic platforms and improved AA detection, it is now recognised that it is not uncommon for SSc patients to express other AA of varying titres (22, 76, 81), and even though it is a rare occurrence, co-expression in various combinations of the three central AAs has been found (22, 81). Less frequent but also considered relatively specific for SSc are anti-U3-RNP/fibrillarin, anti-RNA polymerase I, and anti-U11/U12 RNP. As a consequence of improved AA detection methods and international collaboration efforts to combine cohorts, Anti-Th/To is now also considered to be relatively (but not exclusively) SSc specific (82). This AA is not included in routine diagnostic laboratory testing and it has been suggested that current methods may miss patients positive for Th/To due to the reliance on one epitope, hPop1 (83). Additional important epitopes for this AA are currently being investigated (82) and are described later.

Other AAs that may be associated with SSc but are not specific to the disease include anti-PmScl (75 and 100 epitopes), anti-Ro52/TRIM21, anti-Ku, anti- U1-ribonucleoprotein (U1-RNP) and anti-NOR 90. A summary table (Table 1-5) of all AAs on the Euroimmun Line Immunoassay that were tested in this study is below.

Table 1-5: Summary of AAs on the Euroimmun LIA examined in this study.*

Autoantibody	Sensitivity %	Specificity %	Clinical Features	Subset	Prognosis
CENP (see pp. 35-36)	20-40	66-97	Older at onset, female, PAH, calcinosis, reflux, telangiectasia, ↓mRSS ‡, sicca, anal incontinence.	lcSSc	Favourable
Topo1 (see pp. 37-39)	20.2-40	>98	Severe disease with increased mortality, digital ulcers, joint contractures, ILD, ↑mRSS, proteinuria	dcSSc	Poor
RNAP3 (see pp 39-42)	1.3-20	98	SRC, ↑mRSS, GAVE, temporal association cancer	dcSSc	Poor
Fibrillarin (see pp 45-47)	1.2-14	94.7	African males, younger, ↑mRSS, severe GIT involvement, PAH, PF, increased SRC, myositis, digital ulcers	dcSSc	Poor
Th/To (see pp 43-45)	0.2 -14.3	97.8 – 99.5	PAH or ILD, mild or no skin thickening	lcSSc	Variable
U11/U12 RNP* see p45	3.2	100	Pulmonary fibrosis, GI involvement, milder skin, Raynaud's	lcSSc	Poor
U1RNP (see pp. 48-49)	Caucasians 2-14 African American 13-30 Chinese 16-18	93.9	Younger onset, swollen hands, arthritis/arthralgia, oesophageal dysmotility, myositis, PAH, ILD, ↓mRSS	MCTD	Variable
PmScl (see pp. 49-51)	4-11	64	Gastrointestinal manifestations, ILD	lcSSc	Variable
Ku (see pp.51-52)	4.7	96	Elevated CK, myositis, interval from RP to skin onset short, arthritis, ILD, PH. Mostly mild disease	lcSSc	Favourable
TRIM21/Ro52 (see pp . 52-53)	54.7-57.8	40-60	ILD, poor prognosis, Raynaud's, telangiectasia, trend towards PAH & calcinosis	lcSSc	Less favourable than CENP
Nor90/hUBF (see p. 54)	3.8-4.8	96.7	No clear clinical association however should thoroughly screen for tumours.	Unclear/ Not known	Unclear/ Not known
PDGFR (see pp. 55-56)	0.8	99.5-100	Currently none to report	Not known	Not known

*U11/U12 RNP is included in this summary as it is reported to be specific for SSc; however it is not included on the Euroimmun SSc blot. ‡ mRSS, modified Rodnan Skin Score

Anti-Centromere Proteins A and B (CENP A and CENP B)

Description

Cell replication and division occurs in a dedicated region of the chromosome called the centromere. Without centromere integrity, erroneous cell replication and division can have disastrous consequences such as genomic instability with abnormal cell division as found in autoimmune disease (84, 85) and cancer (86, 87). Within this centromere region specialised chromatin provides the basis for kinetochore assembly where sister chromatids attach. It is within this region that the centromere proteins, CENP A and CENP B, necessary for a functional kinetochore, are located. The first description of anti-centromere proteins was in 1980 (88). CENP A is a 17 kDa histone H3 variant essential for epigenetically marking centromere location (89) to which other proteins dock in the replication process. The anti-centromere immune response is directed against two domains in the N-terminus containing a linear motif (G/A-PR/S-R-R) (90). CENP B is an 80 kDa protein localised in the heterochromatin under the kinetochore (91) where it binds to a 17bp DNA sequence, the CENP B box (92). The major epitope of CENP B maps to the C-terminal part of the protein (amino acids 535–599) (93). The exact function of CENP B is unknown, however it seems to have a role in regulating the formation and action of heterochromatin in centromeres (94) and interestingly unlike CENP A, which is highly evolutionarily conserved, mice can live without CENP B (95).

While autoantibodies to CENP A-F and CENP O have been detected in SSc sera (93) , the primary autoantigens in SSc are to CENP A and CENP B. In CENP B, the first AA to be cloned, there were three independent epitopes identified and these epitopes were recognised by ≥90% of SSc sera containing anti-centromere antibodies (96). CENP B was therefore thought to be the major autoantigen but Mahler et al found that CENP A antibodies (as detected by ELISA) are a more specific biomarker for SSc than antibodies to CENP B (97). Most SSc patients with ACA have antibodies to both CENPs (97-99).

Detection

Serological detection of ACA can precede clinical manifestations of SSc by a number of years and they continue to be expressed throughout the life of the patient (100) with stable titres over time (101). Historically, ACA patterns were detected by indirect immunofluorescence on HEp-2 cells and this is still considered ‘gold standard’ methodology. (73). Further assays have been developed for both research and commercial use. These include ELISA and the LIA with both methods using recombinant proteins expressed in insect cells utilising a baculovirus system (102-104). Alternatively, the commercial synthesis of synthetic peptides (97) and

originally, a cloned fusion protein of the CENP B antigen were used (103). Other detection methods include multiplexed assays such as addressable laser bead immunoassay, planar assays (capturing ligands on a two dimensional array) and the forerunner of the LIA, the dot blot (105). Hanke et al noted when using the LIA that both CENP A and CENP B shared significant associations to clinical manifestations, but were not completely identical and surmised that detection of both antibodies in parallel may slightly increase the diagnostic sensitivity for SSc (104) and Mahler et al found good qualitative agreement and 'remarkably good' quantitative correlation between the CENP ELISAs and IIF on HEp-2 cells (73).

Sensitivity and Specificity

The sensitivity of anti CENP antibodies for SSc is between 20% - 40% with specificity ranging between 66%-97% (97, 106-108). CENPs are occasionally found in other SARDs such as Systemic Lupus Erythematosus (SLE) (2-5%) Sjögren's syndrome (SjS) (5-10%), idiopathic inflammatory myopathies (polymyositis/dermatomyositis) (IIM/PM/DM) (1-3%), MCTD (2-5%), 30% primary biliary cirrhosis (PBC) patients (93, 109) and in less than 3% of healthy individuals (105). Prevalence varies between geographic locations and ethnic groups with Caucasians having the highest representation of this AA (19, 110-112) and African Americans and Asians having the lowest representation (110, 112-115).

Clinical Associations

Patients' positive for CENPs are more likely to be female and are older at disease onset compared to other SSc related AA (19, 111, 116); the exception to this finding lies with Mexican Mestizo patients where no differences in age of onset between the AA subsets are found (117). CENPs are present more often in Caucasians and Mexican Mestizos' than they are in African Americans (19, 110, 116). Prevalence varies throughout Asia with Chinese (113), West Malaysian (118) and Thai patients (119) having a lower prevalence compared to Japan where the prevalence of CENP is similar to Caucasian cohorts (120). In India, CENP positivity accounts for 22.7% of SSc patients (121). The clinical correlates of CENPs include pulmonary arterial hypertension, calcinosis, reflux oesophagitis, telangiectasia and milder skin involvement (19, 111, 116, 117, 122). Other significant clinical associations are sicca symptoms (123, 124) and anal incontinence (125). Although the prevalence rates differ across geographic locations and ethnic groups, the clinical associations remain (126).

Topoisomerase 1

Description

A hierarchical arrangement of tight coils and loops (helical winding or supercoiling) enables chromatin, a combination of DNA and proteins, to be packed into cells. This complex organisation is comprised of DNA wound around specific proteins called histones to form the nucleosome and its appearance is often likened to 'beads on a string'. This hierarchical organisation also allows the binding of enzymes, such as topoisomerases, that are required for DNA replication, transcription and repair.

Topoisomerases introduce temporary single or double-strand breaks in the DNA. A specific type of topoisomerase, Topo1, transiently breaks one strand of the DNA, allowing for adjustments in helical winding. Topo1 is primarily responsible for removing torsional stress generated by processes that leave the DNA overwound or under wound (127). It is a 765 amino acid long enzyme (105-kDa) that contains five distinct regions: the N-terminal domain (amino acids 1–215), core subdomains I–II (amino acids 216–435), core subdomain III (amino acids 436–636), the linker domain (amino acids 637–713), and the C-terminal domain (amino acids 714–765) (128). Several studies have demonstrated that antibodies against Topo1 recognize multiple epitopes on the molecule (129).

In characterising what was thought to be the 70 kDa 'Scl-70' antigen, originally identified by Douvas et al in 1979 in sera from SSc patients (130), Shero et al discovered that the antigen was actually the 100kDa nuclear enzyme Topoisomerase 1 and suggested that the smaller 70kDa protein and another 86kDa protein also detected, were degradation products of the larger enzyme (131). In a separate study, Guldner et al probed purified DNA topoisomerase I isolated from calf thymus directly with the autoantibodies from a SSc patient and from the cross reaction patterns observed with the different antigens and antibodies concluded that DNA topoisomerase I is one of the antigenic components against which autoantibodies are formed in scleroderma patients (132). Thus, the Scl-70 antigen was identified as anti-Topoisomerase 1, however many still refer to it as Scl-70.

Pathogenicity of this AA has remained elusive. Henault el al (133) and Seneccal et al (134) demonstrated that Topo1 complex binding can trigger the adhesion and activation of monocytes which provides a plausible model for a fibrotic cascade, but the complete functional attribute of Topo1 remains unexplained.(135). Kuwana et al took serial measurements of Topo1 levels and has linked these increased or decreased levels with disease activity and have found that if the AA becomes undetectable, prognosis improves (136)

Detection

SSc sera are usually screened on HE-p2 cells by IIF followed by a second test to detect the associated specificities. IIF on HE-p2 cells produce a fine granular to homogeneous staining of the nucleoplasm with or without staining of the nucleoli and chromatin of mitotic cells (137). Topo1 is a precipitating AA and Douvas' original detection of Scl-70 was by immunoprecipitation (130). Methods of detection include immunodiffusion using the Ouchterlony technique (double diffusion) or counter immuno-electrophoresis (CIE) using calf thymus extract, ELISA, LIA or ALBIA using purified native or recombinant topoisomerase 1 fusion protein as the antigen (137). A study conducted by Tamby et al, found that using a combination of IIF, ELISA and an immunoblot increased the sensitivity for the detection of Topo1 from 24.3% to 36.9% using IIF and ELISA (138). In an Asian population, Low et al found good agreement with LIA and ELISA (kappa = 0.97). Another study by Bonroy et al evaluated a fluoro-enzyme immunoassay (FEIA) as an alternative for the combined conventional techniques (IIF on HEp-2000, western blotting (WB), protein radio immunoprecipitation and a LIA) and reported a good overall agreement between combined conventional techniques and FEIA reactivity (kappa>0.800) (139). Shero et al concluded that immunoblotting or solid phase immunoassays were substantially more sensitive for detection than the Ouchterlony test (131) .

Sensitivity and Specificity

Topo1 AA is found in found in 10%–40% of SSc sera (128). Sensitivity for SSc is dependent on the assay used and also the comparative control group (e.g. healthy controls, other CTDs, Raynaud's Phenomenon (RP) and non-SSc relatives). Sensitivity ranges between 20.2% and 40% with a reported high specificity of >98% (108). While high titre Topo1 AA are highly specific for SSc (128), they have been detected in other CTD such as SLE. An early study found that 25% of SLE patients were positive for Topo1 (140) when detected using ELISA, WB and double immunodiffusion (DID). A later comprehensive study using a variety of platforms (ELISA, ALBIA, LIA) found that <5% of SLE sera were positive for Topo1 (128) . This discrepancy is perplexing but no other study has been able to replicate the findings that 25% of SLE patients are positive for Topo1. An answer may lie in the one of five the domains of Topo1 because it seems that the region between amino acid 450 and 600 is a common epitope for the autoantibodies of patients with SSc and SLE, while epitopes localized in the N-terminal domain are recognized mainly by dcSSc sera and those that are found in core subdomains I–II are specific for SLE sera (128). Topo1 are associated with HLA-DRB1, DQB1 and DPB1 and among these markers, DRB1*11 was associated with Topo1 in all ethnic groups, while HLA-DRB1*1101 was found in Caucasians and African-Americans. HLA-DRB1*1104

was found in Japanese and HLA-DRB1*1502 was found in Caucasians and Hispanics (76).

Clinical Associations

The frequency of Topo1 varies according to geographic location and ethnicity. Germany (81) and France (141) have higher frequencies of Topo1 compared to Australia (22), New Zealand (116), Canada (142), the United States of America (USA) (19) and Belgium (24). The most remarkable ethnic association with Topo1 lies with full-blooded Choctaw Native Americans living in south eastern Oklahoma who have the highest prevalence ($469/10^5$) of SSc yet found in any population. A major risk factor for disease is a uniquely Amerindian HLA haplotype (143). Despite these variations, characteristic clinical associations remain (22, 24, 81, 110, 122, 144, 145). Topo1 patients have more severe disease and increased mortality. They develop more digital ulcers, joint contractures, ILD, a high mRSS (19, 22, 76) and proteinuria (76). Topo1 was an independent predictor of pulmonary fibrosis in the EULAR Scleroderma Trials and Research Group database (n=3656) with pulmonary fibrosis present in 60.2% of Topo1 patients.

RNA Polymerase III (RNAP3)

Description

RNAP3 consists of two proteins; a 155kDa protein, IIIA, and a 138kDa protein, IIIB. Together they form a multi protein complex localised in the nucleoplasm (137). RNAP3 assists in the translation of a number of noncoding RNA genes whose products are involved in fundamental cellular processes such as protein synthesis, RNA processing, transcription and chromatin regulation (146). Therefore, the genes transcribed by RNAP3 fall in the category of "housekeeping" genes whose expression is required in all cell types and most environmental conditions. RNAP3 transcription is regulated with cell growth and proliferation and in response to stress. Deregulation of RNAP3 is linked to diseases such as Alzheimer's disease (147), fragile X syndrome (146), and cancer (148, 149). The anti-RNAP3 autoantibody is also one of the three major autoantibodies associated with SSc.

Detection

In 1993 Kuwana et al reported a novel antibody that reacted with RNA Polymerases (I, II and III) (150). Incubation of SSc sera was carried out with (^{35}S) methionine labelled He-La cell extracts and 14/275 SSc sera reacted, precipitating 12 -14 proteins. Purified IgG from these sera then inhibited RNA transcription catalysed by RNA polymerase I, II and III. Immunoblot analysis was carried out and the majority of these sera reacted with a 42kDa or 25kDa protein. In 1998 Chang et al developed an ELISA to detect antibodies against RNAP I, II, and III. In validation studies both

the ELISA and immunoprecipitation of (^{35}S) labelled HeLa cells were used to analyse sera from a large cohort of well-characterized Caucasian SSc patients. They found excellent concordance for the presence of anti-RNAP antibodies between both methods (151). Later in 1998 Satoh et al found that 2% of SLE and Overlap sera also had RNAP activity; this was subsequently revealed to be RNAP2 which is found in patients with SLE or Overlap disease and that RNAPI and RNAP3 are specific for SSc (152).

In 2002 Kuwana et al characterised the immunodominant epitopes on RNAP3 subunits. RPC62 and RPC155 were generated in a bacterial expression system as a series of recombinant fragments. Reactivity fragments were examined by immunoblots and/or ELISA in SSc sera, other CTD disease and in normal healthy control (NHC) individuals. RNAP3 positive SSc sera recognised several distinct epitopes on RPC62 and RPC155 in various combinations, but the fragment encoding amino acids at positions 732-1166 of RPC155 was recognized by all 11 RNAP3 positive SSc sera (153). RNAP3 is now commonly detected using ELISA and immunoblot technologies.

Lastly, RNAP3 titres are known to change over time (154).

Sensitivity and Specificity

After classifying 735 SSc patients for their ANA specificities (fine speckled nucleoplasmic staining with or without nucleolar staining) followed by immunoprecipitation using (^{35}S) labelled HeLa cell antigen, Bunn et al concluded that RNAP1 and RNAP3 AAs represented a discrete serological subgroup (11.7%) (155). A later study found that antibodies to RNAP3 were not consistently associated with such an IIF pattern on conventional HEp-2 cell substrates (156). A major epitope commonly recognized by SSc sera containing RNAP3 autoantibodies was identified on RPC155 when 16/16 SSc RNAP3 positive sera, but not SSc RNAP3 negative sera, were tested using a purified recombinant fragment in an ELISA (153). This epitope was considered by Kuwana as having 100% sensitivity and specificity. In 2005, a new ELISA was developed for detection of RNAP3 antibodies, using a recombinant fragment containing the immunodominant epitope as the antigen source and was compared to the (then gold standard) immunoprecipitation method. This cohort consisted of 522 SSc patients and 516 controls including patients with other connective tissue diseases and blood bank donors. The results of the ELISA showed analytical sensitivity of 91% and analytical specificity of 99% compared with the immunoprecipitation assay. The clinical analysis of the ELISA (with respect to diagnosis) demonstrated that clinical sensitivity was 17% and the specificity was 98% (157) and these results are consistent with other studies (158) (79).

Clinical Associations

The prevalence of RNAP3 varies widely with both geographic location and ethnic background, from 1.4% in Mexico (159) to 23% in the US (160). Table 1-6 demonstrates the geographic and ethnic differences in the prevalence of RNAP3.

Table 1-6 Geographic and ethnic differences in the prevalence of RNA Polymerase III

Country	Prevalence	Ethnicity (Predominant)
Australia	15.3% (161)	Caucasian
New Zealand	20% (116)	Caucasian
Canada	19% (162)	Caucasian
United States of America	23% (110)	Caucasian
United States of America	10% - 13% (110, 159)	African American
France	4%-11%(141, 163)	Caucasian and other
Germany	3.8% (81)	Caucasian
Belgium	6.1% (24)	Caucasian
Italy	3.4%- 7.1% (164, 165)	Caucasian
Mexico	1.4%(145)	Mexican Mestizo
Japan	5.7% - 10.7% (34, 36, 79)	Japanese
West Malaysia	6.5%(118)	Chinese, Malay, Indian
China	1.3% (166)	Han Chinese
Singapore	5.88% (114)	Chinese, Malay, Indian

Clinical associations consistent with RNAP3 are SRC (19, 34, 36, 167), a higher mRSS (34, 79, 81, 168, 169), and more recently, gastric antral vascular ectasia (GAVE) (170-172).

One intriguing association that has been found in a number of studies across geographic locations and ethnic backgrounds is the close temporal association of RNAP3 AA and cancer. This was first reported by Shah et al in 2010 (173). Evaluation of 6/23 individuals that tested seropositive for RNAP1 and RNAP3 found that the duration between SSc and cancer diagnosis fell between 1.3 - 2 years.

While these findings have now been replicated in many other cohorts (161, 164, 174, 175), a well-designed Japanese study did not find any association with malignancy (34). This may be partly accounted for by the low prevalence of RNAP3 in the Japanese population (34).

In 2014 a mutation in the POLR3A gene was identified in patients with coexistent RNAP3, cancer and scleroderma, and analyses of peripheral blood lymphocytes and serum suggest that POLR3A mutations trigger cellular immunity and cross-reactive humoral immune responses in this subset of patients. It was hypothesised that an emerging tumour triggered the immune response and the two conditions

developed synchronously (176), this finding was supported in a study by Airo et al in Italian SSc patients (164). Further to these studies Shah et al sought to determine if autoantibody status and other characteristics are associated with cancer and, a clustering of cancer with SSc onset. (177) They confirmed RNAP3 positivity was associated with a short cancer-scleroderma interval independent of age at SSc onset and that the cancer-scleroderma interval shortened with older age at SSc onset in other antibody groups, particularly among patients with Topo1.

As a result of these findings, it has been suggested that SSc is a paraneoplastic syndrome in some subsets and that these patients should be meticulously screened for malignancies. However it is not known if treating the cancer early would improve the associated SSc (178).

Rarer Autoantibodies Specific to SSc

Th/To

Description

Th/To autoantibodies react with several protein components of the RNA Mitochondrial RNA Processing complex (RNase MRP) and the evolutionarily related Ribonuclease P (RNase P) complex (179). The RNase MRP complex specifically cleaves a number of RNAs including ribosomal mRNAs for the processing of the 5' end of the short form of 5.8S RNA and also mitochondrial RNAs and mRNAs involved in cell cycle control, allowing the cell to exit mitosis (180). The vast majority of RNase MRP is located in the nucleolus (181). The 9 protein components of RNase MRP are Rpp14, Rpp20, Rpp21, Rpp29 (hPop4), Rpp25, Rpp30, Rpp38/40, hPop1 and hPop5 (82). SSc reactivity has been determined with hPop1, Rpp25, Rpp30 and Rpp38 (179, 182-184) with Rpp25, Rpp38 and hPop1 considered as the main autoantigens(82).

Detection

Detection of Th/To was initially difficult due to the lack of commercial assays and although assays are now available, they are still rarely used routinely in diagnostic laboratories. Th/To antibodies show homogenous nucleolar staining in conventional IIF ANA tests, but this is not specific for this antibody. They are conventionally detected by immunoprecipitation of metabolically labelled cell lysates after screening by IIF for ANAs (82, 185) but this is a labour-intensive assay generally only utilised in specialised laboratory settings. A number of other assays have now been developed including a LIA, CLIA, ELISA and IP real time PCR based on either serological cohorts or serum samples identified by IIF staining patterns (82).

Identifying both epitope distribution and a reliable method of detection has become a focus recently as very little is known about the Th/To antigen and some newer assays may not identify patients who have Th/To antibodies demonstrable by IP (82, 184). More recently, assays have been developed based on the Rpp25 and Rpp38 antigens and it has been suggested these may provide greater information with regards to clinical associations (82).

Sensitivity and Specificity

The prevalence of Th/To varies between cohorts, ranging from 0.2% to 14.3% with most between 2.1% - 6.2% (82). The method of detection and the autoantigen utilised has influenced these figures but in addition, there are likely differences in the prevalence within ethnic backgrounds or geographic locations (116, 145, 182, 186). Differences in specificity were also found between methods of detection with LIA at

97.8% and 98.7% (106) using hPop1, CLIA at 99.5% (184) using Rpp25. While the commercial LIA (which detects AA to hPop1) is easy to use and has been validated in a number of cohorts worldwide (22, 106, 166, 187) further studies are required to determine the sensitivity to Rpp25 as a number of studies have shown that this is also a major autoantigen (183, 184) along with Rpp38. Th/To is rarely found in other SARDs and is considered specific for SSc (82, 183).

Clinical Associations

An early study of the Th/To antibody found a decreased frequency of gastrointestinal involvement (188). In 2001, Grunduz et al in an analysis of the University of Pittsburgh Scleroderma Database, found that among 2459 SSc patients, 4/11 patients who were positive for Th/To had PAH and SRC with SRC occurring prior to PAH. Those patients that survived SRC had an increased risk of developing PAH (189). Mitri et al compared Th/To (87 pts) and ACA (306 pts) SSc patients and observed that Th/To patients were younger, had a shorter disease duration at first evaluation, more subtle skin changes, less severe vascular involvement and less distal oesophageal hypomotility. They found the same high frequency of pulmonary arterial hypertension as ACA patients, increased radiographic evidence of pulmonary fibrosis (PF) compared with ACA patients and importantly, reduced survival of Th/To positive patients (190). Some of these findings were replicated by Steen who also found pulmonary hypertension, PF and decreased survival compared to ACA patients (19).

Fisher et al carried out further studies on patients with idiopathic pulmonary fibrosis (IPF) and found 13/25 of IPF patients were positive for Th/To. Furthermore, the Th/To positive patients all had worsening dyspnea; 4/13 had three or more symptoms of CREST and 9/13 met with SSc sine scleroderma criteria (ssSSc) (191). Their survival appeared similar to other patients positive for Th/To without IPF. Fisher et al then reviewed known ssSSc patients with ILD and found 5/6 patients positive for Th/To in their cohort. Common clinical manifestations included scattered telangiectasias, four had RP with abnormal nailfold capillaries, all had reflux, three had oesophageal dysmotility, all had ILD with reduced diffusing capacity of the lungs for carbon monoxide (DLCO), and 5 had pericardial effusion with an elevated pulmonary arterial pressure. An Italian study found similar clinical manifestations, however prognosis was described as ‘excellent’ and pulmonary function impairment appeared mild (186).

The most common clinical associations with Th/To appear to be lung involvement, with mild or no skin thickening and survival yet to be determined. Perhaps stratification of patients can be advanced when larger cohorts are analysed with a

range of sub specificities (anti-Rpp25, anti-Rpp38 and anti-hPop1 antibodies) in an easy to use specific assay.

Anti-U11/U12 RNP

Description

U11and U12 belong to the family of Sm small nuclear ribonucleoproteins (snRNPs), and via protein mediation, they interact to form an 18S complex (192). In contrast to the highly abundant U1, U2, U4/U6 and U5 particles, U11 and U12 are in low-abundance and like the former, are involved in the splicing of pre-mRNAs (192). This snRNP family are also described below under the heading 'U1 Ribonucleoprotein (U1RNP)'. The exact function of the U11/U12 molecule is unknown however it is suspected that they play a role in polyadenylation signalling both upstream and downstream of the AAUAAA sequence. Polyadenylation adds a poly (A) tail to mRNA, which is part of the process that produces mature mRNA for translation.

Detection

Immunoprecipitation of RNP complexes utilising unlabelled K562 or HeLa cells is followed by cross linking of antibodies using human serum IgG to agarose beads. The complex bound beads are washed and resolved by SDS-PAGE and finally, identification of U11 and U12 snRNA is by reverse transcriptase–polymerase chain reaction (193).

Sensitivity and Specificity

In a 2009 study, Fertig et al (193) identified 3.2% consecutive patients over a ten year period had U11/U12 AAs and found them to be 100% specific for SSc.

Clinical Associations

The most serious clinical association with U11/U12 AAs is pulmonary fibrosis. In the study by Fertig et al, 79% of the U11/U12 RNP AA positive patients had lung fibrosis and a 2.25-fold greater risk of death than U11/U12 RNP negative patients (193). None had intrinsic PAH, all had Raynaud's phenomenon, skin involvement was mild and 82% had gastrointestinal involvement.

Fibrillarin (Fib, U3RNP)

Description

Nucleoli are specific subdomains of the nucleus and are present in virtually all eukaryotic cells. They are not a static structure; instead they assemble at the end of mitosis, are active during interphase and disassemble at the beginning of mitosis. It is in the nucleoli that transcription of ribosomal genes (rDNAs), maturation/processing of ribosomal RNAs (rRNAs) and assembly of rRNAs with

ribosomal proteins occur (194). The nucleolus is a multifunctional domain where not only ribosome production occurs but also other functions of the nucleolus, such as cell proliferation control, stress sensing and tumour surveillance, apoptosis, telomere formation, transfer RNA modification and viral life-cycle control. (194). There are three functional components within the nucleolus as identified by electron microscopy, the fibrillar centre, the dense fibrillar component and the granular component. The nucleolar proteins that participate in the early stages of rRNA processing, such as fibrillarin, localise in the dense fibrillar component (195). SSc patient sera that react with this region of the nucleolus and are called 'anti-fibrillarin antibodies' in recognition of this reactivity (196). There is, however, an antigenic complexity to detecting fibrillarin whereby fibrillarin is a component of the many small nucleolar ribonucleic proteins (snoRNPs) that contain box C/D RNAs (197) and although a number of studies have attempted to map the protein for epitopes of the exact binding site of anti-fibrillarin AA, it remains unknown (198) and it is possible that box C/D snoRNPs contain antigenic components other than fibrillarin (197).

Detection

Fibrillarin is a 34kDa basic protein and is an S-adenosylmethionine-dependent methyltransferase of rRNA (197). On IIF anti-fibrillarin AA may show a "clumpy" pattern with bright dots labelling Cajal or coiled bodies in the nucleoplasm that also contain fibrillarin (197). The use of IIF is not always definitive and so further characterisation is required. Over time various methods have been utilised; immunoblotting (IB) (using purified nucleoli) or IP (using radio labelled 35^{S}methionine cell extracts) and even then a positive reaction must be treated with caution as there may be some cross reactivity with other snoRNPs (197). More reliable methods include the use of the cognate fibrillarin cDNA which can be used in an in vitro transcription and translation procedure to generate the full-length protein, which is homologous to that produced in *vivo. This recombinant protein can then be used in an IP assay to detect anti-fibrillarin AA.* IB using native or bacterially–expressed recombinant protein and purified to minimise nonspecific reactions can also be used but one must be mindful that this commonly leads to poor immunoreactivity, possibly due to a loss of conformational epitopes (197). This is important as it is thought that the conformation of the fibrillarin epitope is highly conserved (198). For diagnostic laboratories, there are commercially available kits, including LIA by Euroimmun, (Germany) and an EliA by Phadia, (Sweden) (197).

Sensitivity and Specificity

Fibrillarin AA are most often reported to be specific for SSc, however they have

been detected in SLE, Rheumatoid Arthritis (RA), PM, DM and primary RP patients (199). In SSc they have been found between 0.48% - 14% of patients (199, 200) using a recombinant fibrillarin line blot (201), or an *in vitro* transcribed and translated immunoprecipitation (201, 202) or a HeLa nucleoli immunoblot (203). In an American cohort of 1000 SSc patients, the LIA was recently compared with immunoprecipitation and overall agreement between the two was excellent ($\kappa = 0.966$) with analytic sensitivity and specificity of the U3-RNP LIA 100% and 94.7 %, respectively (196). There is an antigenic complexity to the C/D box snoRNPs and it is important to appreciate that the platform used to detect fibrillarin may not capture all patients as the sera maybe reacting to other proteins within the C/D box (i.e. Nop56 and Nop5/58) (199). Male gender and African descent also greatly influence the presence of this AA (204).

Clinical Associations

Males of African descent have the highest prevalence of fibrillarin compared to females and Caucasians (110, 159, 203, 204). Patients are younger with diffuse skin involvement and high mRSS scores as well as more severe gastrointestinal involvement, heart and lung manifestations including PAH and pulmonary fibrosis (110, 204, 205) and an increased prevalence of renal crisis (196). Amongst patients positive for fibrillarin, PAH was the most common cause of death (205) although one study found no difference in survival between fibrillarin positive versus fibrillarin negative patients (203). Patients positive for fibrillarin also have more muscle disease (205) and experience more digital ulcers (204).

SSc Associated Autoantibodies

U1 Ribonucleoprotein (U1RNP)

Description

Transcription is carried out on pre-mRNA that contains both introns (intervening sequences) and exons (expressed sequences). Introns are removed during pre mRNA splicing, a process that joins exons together to produce mature mRNA (206). The cellular machinery that carries out this process is called a spliceosome and is made up of a set of small nuclear RNAs (snRNAs) and associated proteins. The procedure is energetically expensive requiring hydrolysis of a large quantity of adenosine triphosphate in a two-step process (206). The spliceosome is composed of five different RNP subunits (along with many associated protein cofactors), which can be subdivided into two major classes: Sm and Sm-like snRNAs. U1RNP is an Sm snRNP and is involved early in the exon definition and interacts with U2 to pair the splice sites across an exon (206). U1RNP is not specific for SSc and it is found in other CTDs such as SLE, RA, PM and DM (207). It is considered to be a marker for MCTD where 75%-90% of patients are positive for U1RNP.

Detection

The U1RNP antigen target is a 68kDa (A and C) protein that is detectable by IIF showing a medium granular (137) to coarse speckled (75) pattern. Numerous methods have been utilised over time to detect autoantibodies to U1RNP. These methods include DID (Ouchterlony technique) and CIE using calf thymus extracts, WB using HeLa or MOLT4 tumour cell extracts, EIA,CLIA, LIA or ALBIA using purified native RNP complexes or recombinant protein 68kDa A and C (137) (208), radio IP using ^{32}S-methionine labelled cell extracts, ELISA (209), RNA IP and a protein chip array (210).

Sensitivity and Specificity

U1RNP antibodies are found in 2% - 14% of SSc patients and in about 90% of MCTD patients (76). When comparing CIE, ELISA, IB (using extracts of rabbit thymus and human placenta) with an automated LIA that simultaneously detects nine different AA, the LIA either equalled or surpassed the former assays in identifying U1RNP with a sensitivity and specificity of 87.5% and 93.9% respectively (209). In a separate study of 100 autoimmune myositis patients that compared an ALBIA (native U1RNP and other antigens) with DID and IP (^{35}S methionine-labelled HeLa cell extracts) it was found that DID, IP and ALBIA were equally able to detect U1RNP (211). Kuwana et al examined the role of HLA class II genes in the development of SSc along with a clinical and serological profile in 105 Japanese SSc patients and 104 race-matched NHCs using IIF, DID and IP and found that

U1RNP was associated with DRB1*0401/*0802 and DQB1*0302 and in Han Chinese SSc patients there was an increased frequency of the DQB1*03:03 gene (212). The prevalence of U1RNP varies with ethnicity and this is demonstrated by both Steen (19) and Krzyszczak et al (110) where U1RNP is more common in African Americans (13% and 30% respectively). In Han Chinese SSc patients, U1RNP is present in 18% of patients (166) and 16% of Singaporean Chinese SSc patients (114). In Australia U1RNP is present in 5.5% (76) - 7% (22) of SSc patients and this figure is similar with other predominantly Caucasian cohorts (19, 81).

Clinical Associations

U1RNP patients are younger with the most common clinical manifestations being Raynaud's (211, 213, 214), swollen hands (213, 214), arthritis/arthralgia (211, 213, 214), sclerodactyly (211), oesophageal dysmotility (213), myositis, PAH (213, 215, 216), pulmonary hypertension (22, 214), ILD (213, 214) and milder skin changes (217). Co-expression of other AA maybe a contributing factor to severe disease and U1RNP maybe protective against developing an aggressive disease course (218). This may account somewhat for the great variance in pathogenesis and outcome of patients positive for this AA.

Pm/Scl (PM75 and PM100)

Description

The exosome complex is evolutionarily conserved in most eukaryotes and is involved in many functions including rRNA processing and mRNA degradation (219, 220). A core complex of nine proteins and several other proteins that associate with the exosome in specific subcellular locations or during certain processes constitute the human exosome (219). This autoantigen was first described by Wolfe et al in 1977 when a precipitate was formed from PM patient sera using calf thymus extracts in an immunodiffusion assay and was termed PM-1 (221). It was first labelled Pm/Scl in 1984 when it was found that this autoantigen was present in patients that exhibited features of both polymyositis and SSc. In 1990, two proteins were identified, PM75 and PM100 (based on their molecular weights), that were found to be the main antigenic targets of this macromolecular complex (219). It is not exclusive to SSc and is also detected in patients with polymyositis and dermatomyositis though their highest occurrence are in SSc overlap syndromes (219).

Detection

Screening on IIF (HEp2 cells with calf thymus extract) shows a typical nucleolar staining pattern with a fine specking of the nucleoplasm (76) requiring confirmation with IB (using extractable nuclear antigens), IP (with radioactively labelled cell extracts), ELISA, LIA (using recombinant proteins) and protein chips (recombinant proteins) or ALBIA (219). Most ELISA and LIA use recombinant proteins expressed in *E.coli* or insect cells and are the predominant method of detection (222).

Sensitivity and Specificity

Pm/Scl autoantibodies are found in 4-11% of SSc patients (76) with the majority of Pm/Scl positive patients having antibodies to PM75 (most common target overall) or PM100 (with the primary target being PM1-alpha) (76). Approximately 64% of Pm/Scl positive patients are also positive for Rrp4, another of the core components of the exosome (220).

Clinical Associations

A comprehensive tri-nation study (Australia, Canada and USA) on 1574 SSc patients focusing on the clinical features associated with monospecific reactivity to PM75 and PM100 using LIA (baculovirus system using insect cells) (222).

In this study 16 (1%) patients were monospecific for PM75, 11 (0.7%) were monospecific for PM100 and 22 (1.4%) were positive for both to the exclusion of all other AA. Of note there were 26 patients in the entire cohort that expressed positivity to PM75 and PM100 as well as other AAs. Of interest was the low prevalence of inflammatory myositis in either monospecific group, PM75 (7.7%) or PM100 (0%), whereas the highest prevalence of inflammatory myositis (36%) was among the subjects expressing PM75 and PM100 antibodies and another AA and/or AAs. The analysis showed that both monospecific PM75 and PM100 had more calcinosis than other SSc AAs but clinical differences were then found in isolation between each of these AA and other SSc AAs. PM75 was frequently associated with gastrointestinal manifestations (gastroesophageal reflux disease 87.5%, dysphagia 68.8%, requiring antibiotics for bacterial overgrowth 14.3% and anal incontinence 28.6%), ILD was common (50.0%), second only to the Topo1 group (56.1%) and pulmonary hypertension (21.4%) was most frequent in PM75. In unadjusted survival analysis, PM100 was associated with a significantly better survival compared to RNAP3 and there was a trend toward better survival compared to Topo1 (222). As these authors noted, conceivably there is an interactive effect with individual AAs, with each additional AA contributing to or protecting against a particular clinical characteristic because all other studies on PmScl bar one (223) did not exclude other AA positivity or investigate monospecificity of the PmScl AAs (81,

200, 224-227) and although generally there are similarities with clinical manifestations, they are all subtly different. Monospecificity is rare, and so ongoing international collaborations with different ethnicities are required to confirm these findings and to determine differences between geographic location and genetic background.

Ku

Description

The Ku antigen is a nuclear, non-histone, heterodimeric multifunctional protein made up of two subunits, Ku70 and Ku80 with specific binding affinity for DNA and less so for RNA (228). Its roles include repair of DNA double stand (dsDNA) breaks, V(J)D recombination of immunoglobulins and T-cell receptor genes, DNA replication, transcription regulation, structural regulation of telomeric ends, regulation of heat shock induced responses and a role in the G2 and M phases of the cell cycle (228). The functionality of the Ku antigen lies with its ring like structure that encircles the DNA duplex providing a docking platform for DNA dependent protein kinase and a ligase complex that facilitates end processing and ligation of DNA broken ends (229). Ku is involved in non-homologous end joining (NHEJ) where breaks are repaired due to DNA damage (ionising radiation, oxidative stress or chemical exposure) but it also has the potential to incorrectly join DNA sequences resulting in chromosomal translocations, deletions and insertions. In telomeres, an absence of Ku results in telomere shortening and increased rates of telomere end fusions and ultimately overall genomic instability (230).

Detection

IIF on HEp-2 and other tissue culture cells typically show a fine speckled staining pattern of interphase nuclei and nucleoli (228). Ku antibodies react with natural or recombinant Ku70 and Ku80 and were initially defined by DID or CIE and more recently by IP, ELISA (229) CLIA and LIA (228). Until recently, anti-Ku was rarely tested in commercial laboratories and little is known about its clinical associations with the newer platforms (228). It has been reported that the LIA has problems with quantitation of autoantibody reactivity and analytical sensitivity (70). Historically, the ‘gold standard’ for detection is IP, although ELISA has been reported to have comparable sensitivity (229).

Sensitivity and Specificity

Anti Ku is not specific for SSc although it is often found in people with CTDs. A cohort of 484 SARD patients were tested for Ku compared to NHCs and disease controls using a CLIA platform (research use only assay). The results demonstrated that SLE had a higher prevalence (9.8%) of Ku than SSc patients (4.3%) who had

SSc/SLE/AIM overlap disease (228). Sensitivity and specificity for Ku in scleroderma appears to vary with the type of platform used to detect the AA as well as the patient's genetic background with reactivity to either/both subunits (228, 231, 232). In Italian cohort of 210 patients using LIA the sensitivity and specificity for Ku AA were 4.7% and 96% (200), while same LIA in an Australian cohort of 129 patients identified 5% were Ku positive. While it appears that the prevalence of Ku in SSc is similar in some cohorts it is evident that further research is required to develop an assay that is able to detect various epitopes of Ku in patients of different genetic backgrounds.

Clinical Associations

Ku is more often associated with SLE and myositis related conditions (PM, DM, AIM) than with SSc (233). Patients that have SSc and are Ku positive are often classified with an overlap condition and their disease progression is different to that of patients with traditional SSc AAs (233, 234). Anti-Ku positive patients in a German cohort experienced myositis and an elevated creatine kinase and the interval between RP onset and skin onset was found to be the shortest of all the SSc associated AAs (0.7 ± 0.7 years) (234). Further clinical associations (depending on genetic background and detection methodology) include arthritis, ILD and PAH with a recent finding that there are neurological manifestations in connection with CTDs (137). Overall, those positive for Ku appear to have a more benign disease course and a good response to immunosuppressive therapy (137).

TRIM21/Ro52

Description

TRIM21/Ro52 belongs to the TRIM superfamily of proteins. characterised by their highly conserved domain, the RING/Bbox/coiled-coil tripartite motif (235). TRIM21/Ro52 is involved in regulation of the innate immune response specifically as an E3 ubiquitin ligase by recognising, binding and labelling the target molecule for degradation in the ubiquitination process (235). TRIM21/Ro52 has the ability to regulate downstream signalling of various pattern recognition receptors such as the NF-κB, TGF-β and interferon response (236). Interestingly, recent research has revealed that TRIM21/Ro52 antibody-mediated protection extends to the cytosolic compartment of cells, a process that was thought to only occur in the extracellular environment (237). TRIM21/Ro52 is found mostly in the cytoplasm, however it is translocated from the cytoplasm into the nucleus on interferon alpha stimulation and it has also been reported to be translocated to the cell surface in apoptotic or stressed cells (235). As an autoantibody in SARD, TRIM21/Ro52 is primarily found in SLE and SjS, but also in SSc, PM, DM, IIM, MCTD and RA (137).

Detection

TRIM21/Ro52 is a 52 kDa protein identified by Chan et al by isolating cDNA clones from human HepG2 and MOLT-4 cell cDNA libraries. In addition, they identified that TRIM21/Ro52 (or as it was then known, SSa-Ro52) was a discrete protein and not in a complex with another protein SS-A Ro60 (238) The identity of this protein was established in three ways; by the specificity of the antibody affinity purified from the recombinant protein, the reactivity of the purified recombinant protein with prototype SS-A/Ro sera in immunoblot and ELISA, and two-dimensional gel co-migration of MOLT-4 cell 52-kD protein and the recombinant protein (238). The TRIM21/Ro52 autoantigen can be detected on a variety of platforms including EIA, LIA, CLIA, ALBIA (137) or ELISA (239) with purified native or recombinant antigen. False negatives and positives may occur if using an SS-A ELISA that uses a mixture of both antigens in a single assay (240).

Sensitivity and Specificity

In one cohort of 89 ENA positive and 90 randomly selected ENA negative patients, a CLIA and an ELISA were evaluated with both platforms using an antigen composition of recombinant insect cells. In addition (and using the same methods), 64 SSc patients, 363 other SARD patients and 605 disease controls were evaluated. The results showed that 39.3% of the ENA positive group were positive for TRIM21/Ro52 and 0% in the ENA negative group. In the SSc, SARD and disease comparator group sensitivity for SSc patients using the CLIA and ELISA were 54.7% and 57.8% with specificities of 95% and 97.8% (239). A study in an Asian SSc and SLE population compared a LIA with an ELISA. In the LIA when comparing SSc with SLE, the specificity was 63% with a positive predictive value of 60% and a negative predictive value of 40% (144). This is a potential problem when comparing SSc with SLE in this (and possibly other) population(s). TRIM21/Ro52 is the most common co-expressed AA in SSc and occasionally it is expressed monospecifically (162). In a large tri-nation study (Australia, Canada and USA), of 1574 subjects, 6.5% were monospecific for TRIM21/Ro52 and 20.6% had TRIM21/Ro52 co-expressed with other SSc AA (241). In conclusion, TRIM21/Ro52 can be expressed both monospecifically and in combination with other AAs, and is not specific for SSc.

Clinical Associations

In the tri-nation study, TRIM21/Ro52 was significantly associated with ILD and overall had a poor prognosis (241) with the ILD result mirroring that of a Canadian study (162). The association with lung fibrosis was also found in a Norwegian cohort of MCTD patients (242).

Human Upstream Binding Factor (hUBF /NOR-90)

Description

In 1934 while studying the genetics of *Zea mays,* Barbara McClintock proposed the 'nucleolus is organized in the telophase through the activity of ... the **n**ucleolar-**or**ganizing (NOR) body' (243). In 1987 Rodriguez-Sanchez et al identified an SSc patient whose serum contained a high titre of IgG antibodies that stained the nucleoli in a pattern of independent tiny spots. Immunoblots were then performed with serum from this patient on isolated nucleolar substrates and they identified a protein of approximately 90 kDa (244). Then in 1991, Chan et al reported that by using a cDNA clone of the NOR-90 antigen this probe identified an alternative form of the human upstream binding factor (hUBF) (245). Subsequent results from immunoprecipitation assays demonstrated that NOR-90 antibodies recognised hUBF/NOR-90 (245).

Detection

IIF on HEp-2 cells shows granular or speckled staining of the nucleoli and some dots in the chromatin of mitotic cells (137). Other methods of detection include WB using HeLa, HEp-2 or MOLT4 cell extracts with well characterised NOR-90 antibody or serum, IP of recombinant protein expressed in Sf9 cells or LIA with recombinant hUBF(137).

Sensitivity and Specificity

In one cohort of 210 Italian SSc patients the sensitivity and specificity for NOR-90 was 4.8% and 96.7% respectively (200). This surprising finding in specificity occurred because nine of their patients were positive only for this AA. Generally, hUBF/NOR-90 is considered less specific for SSc, found in < 5% of SSc patients (246). In a cohort of Japanese patients with rheumatic diseases, anti-NOR-90/hUBF was investigated using an IB of recombinant fusion proteins expressed from several cloned cDNAs encoding the NOR-90/hUBF antigen. Of the 91 sera tested, 9 were positive for NOR-90/hUBF (9.9%). Seven of the patients had SjS, 4 had concomitant RA, 1 had concomitant SSc and 2 (2.2%) had SSc in isolation (247). NOR-90 are seen in a variety of inflammatory rheumatic diseases with about one third of these patients in the SSc spectrum but they are also found in other conditions such as alcoholic liver cirrhosis and hepatocellular carcinoma (137).

Clinical Associations

NOR-90 is a rare autoantibody and there are no clear clinical associations. If NOR-90 AAs are detected, then other clinical symptoms should be thoroughly investigated to determine if a SARD or tumour is present.

Other Autoantibodies

A number of other AAs have been reported in SSc which at this time are felt to have limited wider clinical significance, either due to lack of specificity or there may be difficulty in obtaining a commercial diagnostic assay and they have been extensively reviewed elsewhere (65, 76). A summary of those AAs that are not included in this study but have been detected in SSc are in Table 1-7. Lastly, the platelet derived growth factor receptor AA which is included in this study, is discussed in greater detail below.

Platelet derived growth factor receptor (PDGFR)

Description

Platelet derived growth factor (PDGF) is a growth factor involved in the regulation of cell growth and repair, particularly in angiogenesis, but also in other developmental processes (248). PDGFRs are high affinity cell surface tyrosine kinase receptors for the PDGF family. In SSc, PDGFR autoantibodies were hypothesized to have a pathogenic role because PDGFR expression is increased by TGF-β signalling and binding of PDGFR AA to the PDGFR ligand results in amplification of the Ras-extra-cellular signal-regulated kinase 1/2-reactive oxygen species (ROS) cascade, leading to enhanced collagen production (76). To date, there is conflicting data regarding the existence, stimulatory activity and SSc specificity of PDGFR AAs (249-251)

Detection

Perhaps some of the controversial findings from PDGFR AAs may be explained by the use of varied assays, their complexity and the difficulty in reproducing results from initial experiments by Baroni et al (252) who used mouse embryonic fibroblasts with or without PDGFR inhibitors and characterised the AA by IP, IB, and absorption experiments. They found that PDGFR antibodies induced tyrosine phosphorylation in normal fibroblasts with an increase in reactive oxygen species and production of α-smooth muscle actin and type I collagen production which supported a pathogenic role of PDGFR (253). Subsequently, Loizos et al (250) used electro-chemiluminescence binding assays to detect binding of purified immunoglobulins to PDGFR and found that it wasn't specific to SSc and was found in only 33% of SSc sera. They also found PDGFR was present in 34% of NHCs.

Classen et al (249) generated a 32D mouse cell line transfected with human PDGFRα and PDGFRβ to assess the PDGFR agonistic activity of purified IgG. PDGFR activation was tested using 4 different sensitive bioassays, i.e., cell proliferation, ROS production, signal transduction, and receptor phosphorylation and found that neither PDGFRα nor PDGFRβ was specifically activated in any of the

tests. Classen concluded that the results from their study raised questions in regards to the existence of agonistic autoantibodies to PDGFR in SSc. A recent study by Moroncini et al (254) concluded that epitope specificity determines pathogenicity and detectability of anti-PDGFRα AAs after generating different recombinant human PDGFRα AAs from B cells derived from an SSc patient then using a direct or competitive ELISA to detect all serum PDGFRα AAs. From this study, Günther et al proposed that there is a heterogeneous set of anti-PDGFRα AA involved in SSc pathogenesis that shows distinctive functional properties (135). Lastly in a complex experiment to demonstrate that PDGFR AAs induced skin fibrosis in vivo, Luchetti et al (255) bio engineered skin from different SSc clinical subsets and NHCs and then grafted the skin onto mice and injected SSc IgG into the NHCs. In addition, nilotinib (a tyrosine kinase receptor inhibitor of PDGFR) was administered to some NHC mice. The experiment generated significant dermal collagen accumulation in the mice without nilotinib, and the nilotinib mice resembled the NHC mice in that they had less skin thickness, collagen deposition and dermal vessel rarefaction. Finally, they injected PDGFR stimulatory and non-stimulatory monoclonal antibodies into the NHC mice as nilotinib has been shown to inhibit other fibrotic molecules such as TGFβ. The mice with stimulated PDGFR antibodies developed a SSc phenotype measured by histologic features and vessel rarefaction.

Fritzler and Choi comment on this experiment and explain that there are many factors involved in such vascular changes such as 'ligand–mediated dimerization and other requirements for cell signalling which were largely unaddressed…' (253). Much work is still to be done to clarify the position of anti-PDGFR as a pathogenic AA in SSc.

A commercial LIA is available using recombinant PDGFR expressed in mammalian cells (256), but to date, as seen in the section below, there are few positive anti-PDGFR sera in the cohorts tested by this assay.

Sensitivity and Specificity

This LIA has been used in a number of cohorts varying in geographic locations and ethnic background and the results are as follows; in an Asian Singaporean population no patients had this AA (144), in unpublished data, less than 6% of a Canadian SSc cohort of 800 patients were positive (76), in a New Zealand cohort of 60 SSc patients none had PDGFR AAs (116) and in an Italian cohort of 210 patients the sensitivity was 0.95% (2/210) (200). PDGFR AAs have been found in NHCs (249) as well as SLE patients (251) and appear to be a very rare AA in SSc.

Clinical Associations: At present there are no clinical associations to report.

Table 1-7: Summary of lesser known AAs found in SSc patient sera

Autoantibody	% Frequency in SSc	Clinical Features	Subset	Prognosis
B23/nucleophosmin/numatrin	-	Mostly associated with SLE or variants PAH, hepatocellular carcinoma and other malignancies. (257)	lcSSc	Unknown
Nucleosome, #histone, §high mobility group (HMG) proteins	#Anti-histone 16%-29% in SSc §HMG detected in ~33% of SSc in (258)	#PF, cardiac and renal. If coincident with CENP then severe pulmonary and vascular disease. (76)	Unknown	#Decreased survival § Severe disease
Nuclear envelop and nuclear pore complex	Rare	Unknown	Unknown (potentially linear scleroderma?) (259)	Unknown
Anti-Endothelial cell antibodies (AECA)	44-84% SSc but also other SARD	Digital scars/ulcers, severe RP, PF and PAH, peripheral vascular injury. Linked to fibrillarin AAs (260)	Both	Decreased survival
Anti-Fibroblast antibodies	26-58% of patients in (261)	Potentially related to severity of skin disease, ILD and PAH	Highest prevalence dcSSc	Unknown
Anti-Mitochondrial, anti-Sp100 combined. Anti-gp210 (76)	Varying, depending on study: PBC in SSc ~2%- 5%	Primary biliary cirrhosis (PBC)/autoimmune liver disease. Less calcinosis and telangiectasia	PBC and/or lcSSc	Unknown
Anti-neutrophil cytoplasmic antibodies (ANCA), PR3, MPO (76)	~5%	Associated with ANCA associated vasculitis.	No conclusive association.	Unknown
β2 glycoprotein I (anti-β2GPI) and anti-cardiolipin (aCL), Anti-phospholipid (aPL) antibodies (76)	~5%-41%	aPL & aCL/β2GPI – PAH, digital ischemia or severe RP. β2GPI with digital loss. aPL with miscarriage in SSc	No conclusive association	Unknown
GW body antibodies	~14%	Potentially associated with PBC/SSc patients (76)	Unknown	Unknown
Survivin antibodies (76)	41% in 1 study	Significantly longer disease duration	No conclusive association	Unknown
Activating transcription factor-2 (76)	Unknown	Significantly longer disease duration, decreased vital capacity and DLCO. (262)	Unknown	Unknown
Glycan antibodies (76)	14.9% in one study (263)	PAH	Unknown	Unknown
Angiotensin II (AT1R) and endothelin-1 (ET AR) (76)	In SSc AT1R, 85.1% sensitivity and 77.9% specificity ET AR, 83.7% sensitivity and 77% specificity (264)	Associated with severe and early disease, PAH, lung fibrosis, digital ulcers, renal crisis. Predictive for SSc mortality and potentially biomarkers for assessment of disease progression or response to therapy	dcSSc	Unknown
Annexin V antibodies (76)	In one cohort of 20 SSc patients, 75% had Annexin-V (265)	Vascular damage	Either	Unknown
Fibrillin-1 antibodies (76)	>50%	Uncertain	lcSSc, dcSS, & MCTD in certain ethnic groups	Uncertain
Matrix metalloproteinases (MMP). MMP1 and MMP3 antibodies (76)	49-52%	Potentially reflects the severity of SSc fibrosis in skin, lung and renal blood vessels (266)	Mostly dcSSc	Unknown
Cyclic citrullinated peptide (CCP), rheumatoid factor and anti-agalactosyl IgG antibodies(76)	CCP in SSc 2.6%-12%	CCP- Arthralgia SSc/RA overlap RF – erosive arthritis	Either	Unknown

Autoantibody	% Frequency in SSc	Clinical Features	Subset	Prognosis
Tissue plasminogen activator antibodies (76)	Unknown	Increased frequency of PAH (267)	Increased frequency lcSSc (267)	Unknown
Peroxiredoxin I antibodies (76)	In one cohort (n=70), 33% positive (268)	Longer disease duration, PF, cardiac involvement, increased IgG and ESR rates. Correlated with renal vascular but not .skin damage. (268)	Either	Unknown
Interferon-inducible gene 16 (IFI16) antibodies (76)	18% - 21%	Longer disease duration and decreased DLCO, Vasculopathy and DUs. Changes in mRSS(269)	Greater frequency in lcSSc (269)	Unknown

Unresolved Issues in Systemic Sclerosis

Autoantibodies appear to be an integral component of autoimmune disease and while much progress has been made in understanding the expanding repertoire of autoantibodies associated with SSc, important fundamental questions remain. Firstly, are the SSc autoantibodies pathogenic? Secondly, over time, how stable are the AAs? Thirdly, is the concept of AAs being 'mutually exclusive' redundant?' Finally, can AAs be used to better identify disease associations? While the answers to these questions are still uncertain, emerging research built on improved technologies are offering exciting possibilities for AA research and will hopefully clarify these significant points.

Are SSc autoantibodies pathogenic?

At present the pathogenesis of SSc remains enigmatic. As Fritzler and Choi (253) explain:

'…*the prevailing paradigm has been that the pathogenesis of SSc is largely related to immune dysregulation, vasculopathy, and uncontrolled extracellular matrix production in the context of a T cell and cytokine/chemokine/growth factor-mediated process…*'

Since Henault el al (133) and Senecal et al (134) first demonstrated that Topo1 complex binding can trigger the adhesion and activation of monocytes, therefore providing a plausible model for a fibrotic cascade, the question of pathogenicity of this AA has persisted. In support of the pathogenic hypothesis, Kuwana et al took serial measurements of Topo1 levels; linked these increased or decreased levels with disease activity and found that as the AA becomes undetectable, prognosis improves (136). Despite these findings, the complete functional attribute of Topo1

remains unexplained (135).

Anti-PDGFR antibodies are controversially also reported in some experiments to be pathogenic (254, 255, 270) but not in others (249, 250).

For patients that are in an early disease phase and are yet to be diagnosed and classified as having SSc, the strongest predictor of disease progression is the presence of an AA (271, 272). If patients who are not AA positive do not progress to SSc or another CTD, this would suggest that either treatment/s were effective or the absence of an AA impeded disease progression. It is hypothesised that other AAs may be protective as is the case with U1RNP (273), although further work is required as not all subjects with this AA do well (23).

Many more recently identified AAs are thought to play a pathogenic role in both the initiation and development of SSc and in particular in the progression of vasculopathy and fibrosis (218). These include several of the newly discovered AAs in SSc in Table 1-7 (pp.51-52) (AT_1R, ET_AR, IFI16, Anti-Fibroblast antibodies, MMP1 and MMP3) and others such as intracellular adhesion molecule, oestrogen receptor α, methionine sulfoxide reductase, type 3 muscarinic acetylcholine receptor and anti-PDGFR (253). Future studies on AAs that are considered pathogenic or 'functional' need to capture patients early in their disease course to clarify pathogenic pathways so that therapeutic targets can be identified and interventions can be developed. However, critical for early disease detection is a greater understanding of how very early SSc presents, not only clinically and serologically, but also at the molecular and importantly the proteomic level.

Although pathogenicity of scleroderma AAs remains uncertain, there is much scope for the development of better AA detection and for the development of assays to determine functionality (253). With the advent of new technologies and further understanding of the repertoire of AAs and their role they play in SSc, the question of pathogenicity may become clearer.

How stable are AAs?

AA titres are thought to be stable over the course of disease (246); however experiments measuring Topo1 titres have shown fluctuation occurs over time in association with an changes in disease activity as measured by the mRSS (274), lung involvement and survival rates (136, 275).

Fewer studies are available on the stability over time of RNAP3. One small study (n= 6) showed that RNAP3 levels (as detected by ELISA) increased early in the disease course and then decreased with the fluctuation of RNAP3 AA levels closely

correlating with skin score (157). Two patients developed renal crisis early in the disease with a synchronous rise in their RNAP3 levels.

CENP appears to be relatively stable throughout the disease (101) and the stability of other AAs is unknown. To determine the stability of AAs and if pathogenicity is related to the level of AAs in patient sera, further longitudinal studies in multi ethnic and varying geographic locations are required.

Are autoantibodies mutually exclusive?

This question is generally asked of the two SSc primary AAs, CENP and Topo1, although some studies have included RNAP3 (276). The literature oscillates between two hypotheses; there is mutual exclusivity between the two central AAs, CENP and Topo1 (276-279) *or* co-expression of these two antibodies exists as co-expression of AAs exist in other autoimmune diseases (280, 281). The most recent SSc AA research using multiplexed immunoassays such as the LIA, ALBIA and CLIA show that multiple AAs can be co-expressed in a single patient (76). Co-expression of the central AAs, essentially CENP and Topo1 is uncommon, yet it does occur. Co-expression with one of the other SSc associated AAs is more likely, particularly TRIM21/Ro52 (76, 282).

As technologies have evolved into multiplexed arrays and further epitopes on individual AAs are mapped, more AAs are found to be co-expressed. Previously, the laborious task of performing individual assays was necessary to detect individual AAs. The key point here is that meticulous attention to the standardisation of these new assays is required to ensure that cross reactivity or false positives/negatives are minimised. Finally and most saliently, the clinical and pathological significance of co-expression of two or more AAs is yet to be elucidated.

Can AAs be used to better identify disease associations?

Ultimately, the question must be asked if the AAs can be used to better identify disease associations. If for example, AA titre is linked with disease course and outcomes, then other biomarkers may be useful to predict patient outcomes or other clinical involvement. A Canadian study investigated serology and skin involvement with survival in dcSSc and lcSSc patients with both subsets having either Topo1 and ACA positivity and found that ILD was associated with serological status more so than skin subset. However to add complexity, survival was associated with both serological status as well as skin subset (52). Therefore, at least among Topo1 positive patients, a lower skin score is associated with a reduced likelihood of developing ILD and increased survival. A study by Cottrell et al also concluded that defining the pattern of skin involvement together with the autoantibody status was of

greater prognostic value in predicting the risk and severity of restrictive lung disease as well as overall survival in SSc as compared with using either variable in isolation (283).

Conclusion

Scleroderma is a heterogeneous disease associated with significant morbidity and mortality.

Treatment options remain limited but it is widely accepted that early disease detection and sub-classification is critical to identify those patients who may benefit from experimental and other therapies before significant damage has accrued. The newer classification criteria for SSc will hopefully aid earlier diagnosis of SSc patients but sub-classification is also critical as current treatments are associated with significant toxicities and should not be applied to all those with the disease. Previously sub-classification has been limited to the degree of skin involvement although it is widely recognised that AAs provide useful clinical information.

The primary SSc AAs have been well characterised and have recognised clinical associations across different geographic locations and ethnic backgrounds. Consideration must be given to the platform used to detect AAs as well as the epitope and vector used in the assay. While standardisation is crucial, patient background must be taken into consideration and so continuous improvement and validation across patients groups is important.

With the advent of LIA and other platforms it is now possible to simultaneously test for a number of AAs, however the relevance and clinical interpretation of multiple AA positivity is unknown, therefore further characterisation in large well described disease cohorts is important.

It has been demonstrated that SSc AAs predate overt disease and are generally stable over time, that is, it is rare for them to disappear. As a result of this trait, they are a useful biomarker for early disease classification and, if used with other validated biomarkers, may be useful in disease stratification and prognosis.

Aims & hypotheses for this study

The primary aim of this study is to determine the frequency of, and the relationships between, SSc related autoantibodies as well as their clinical associations in a well characterised Australian cohort using a commercially available assay.

In detail the aims for this study are;

i. To conduct an AA profile on this well characterised Australian cohort.
ii. To explore the utility of AAs as biomarkers.
iii. To define AA subgroups based on clinical associations for prognostication and to better identify patients for clinical trials.
iv. To investigate the usefulness of AAs as a stratification system.
v. To assess if AAs can be used to better identify disease associations.
vi. To describe clinical associations with the primary and rarer SSc associated AAs.
vii. To establish the frequency of monospecific and multiple AA positivity in an Australian cohort.
viii. To explore unique clinical associations in subgroups (where possible) of monospecific and multiple AA presentation.
ix. To explore the concept of mutual exclusivity of CENP and Topo1 AAs.
x. To compare the reliability of the Euroimmun LIA with other commercially available assays. Specifically, where results differ, to examine the clinical associations of these patients to see whether the test result supports the patients disease sub classification.
xi. To determine the frequency of PDGFR in an Australian SSc cohort.

The hypotheses for this study are:-

i. The LIA can be used to sub-classify patients with SSc.
ii. Multiple AA positivity is common in SSc.
iii. Important AAs and/or combinations of AAs will provide valuable clinical and serological information, independent of clinical assessment
iv. AA titre will allow hierarchical assessment where multiple AAs are detected.
v. Clinical associations can be explored with the rarer SSc AAs in an Australian SSc cohort.
vi. Although rare, co-expression of Topo1 and CENP forms a unique clinical subgroup.
vii. AA negative patients have a unique clinical phenotype which differs from other subsets of SSc.
viii. Determine if improvements can be made in the LIA AA selection.
ix. The LIA has similar sensitivity and specificity to other commercially available assays used in the ASCS.
x. PDGFR will be very rare in this cohort.

It is with these hypotheses in mind that this study was undertaken and it is hoped that the outcomes from this study will contribute to the knowledge and utility of autoantibodies as biomarkers in systemic sclerosis.

CHAPTER 2
METHODOLOGY

The Australian Scleroderma Interest Group (ASIG)

The ASIG is a national, multidisciplinary group of physicians and scientists interested in research that furthers the understanding of scleroderma and seeks to improve outcomes for patients. The ASIG established the Australian Scleroderma Cohort Study (ASCS) in 2007 as a prospective multicentre study to conduct an annual screen in SSc patients for lung and heart complications and to collect biological samples (cells, DNA and sera) from consenting patients to be enrolled in a longitudinal observational cohort for ongoing research projects. Patient consent was obtained in writing after the provision of information concerning the scope of research projects and individual personal information required for entry into the cohort study. The database is managed by the Database Committee who oversees the maintenance of the existing ASIG clinical database with administration assistance from a Project Officer. The Terms of Reference including structure, governance and scope can be found in the appendices, 'Australian Scleroderma Interest Group Terms of Reference (2013)'.

Study Design and Ethical Approval

This is a cross sectional study of Australian SSc patients enrolled in the ASCS database. Ethics for this study were approved by the ethics committee of the participating centres as well as by the Southern Area Clinical Human Research Ethics Committee at Flinders University. This study 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 (284), and in accordance with the Declaration of Helsinki (285).

Patient Population

At the time of testing there were 1,139 patients enrolled at 8 centres specialising in the care of patients with SSc: Royal Adelaide Hospital (Adelaide, South Australia), St. George Hospital (Kogarah, New South Wales), Sunshine Coast Rheumatology and Prince Charles Hospital (Brisbane, Queensland), St. Vincent's Hospital and Monash Health (Melbourne, Victoria), Royal Hobart Hospital (Hobart, Tasmania) and Royal Perth Hospital (Perth, Western Australia). SSc patients were admitted to the ASCS database based on one of three significant conditions;

i. The patient fulfilled the ACR 1980 criteria (286), or
ii. The patient fulfilled the LeRoy and Medsger criteria for early SSc (287), or

iii. The condition was diagnosed according to the expert opinion of the patient's treating physician.

A further patient group is also included in the ASCS database and serum repository, those patients that are considered to have MCTD, diagnosed using Sharp's Criteria (288), or again, by the expert opinion of the treating physician.

The ASCS Serum Repository, located at The Queen Elizabeth Hospital, Woodville South Australia, made available 526 serum samples for this study. Demographic data collected and utilised in this study include age (chronological, disease onset Raynaud's and first non Raynaud's symptom), gender, disease subset, ethnicity and fulfilment of ACR or Medsger criteria. Data for this study were censored on 7th June 2013. The patient sera used in this study were not taken from inception samples. The new 2013 ACR/EULAR criteria had not been published at census date for this study, however for completeness, the new ACR/EULAR 2013 criteria have been retrospectively assessed and included on the 505 SSc patients. Full demographical results are in Chapter 3, Results, Table 3-1, p.78.

Disease manifestations were defined as present if they had occurred since the time of diagnosis or, for continuous variables including the modified Rodnan skin thickness score, the highest ever recorded value since time of enrolment in the ASCS. Study variables and their description can be found in Table 2-1.

Table 2-1: ASCS Clinical and serological variables

Variable	Description
Disease duration	Calculated since first non-Raynaud's symptom
Raynaud's phenomenon	Characterised by triphasic colour changes, date onset
Digital ulcers	Defined as denuded areas with defined borders and loss of epithelialization, epidermis, and dermis; excluded fissures, paronychia, extrusion of calcium) either on the volar or dorsal aspects of the fingers
Digital gangrene	Diagnosed clinically by the treating physician
Digital amputation	Ever
Nailfold capillary dilatation	Capillary measurements include dilatation only.
Telangiectasia	Any clearly visible mat-like telangiectasia visible on the face, limbs, chest or abdomen.
Calcinosis	Diagnosed clinically by the attending physician
Tendon friction rubs	Diagnosed clinically by the attending physician
Joint contractures	Diagnosed clinically by the attending physician
Synovitis	Diagnosed clinically by the attending physician
Modified Rodnan Skin Score (mRSS)	The mRSS measures skin thickness on a score of 0 (no thickness) to 3 (severe thickening) in 17 locations on the body.
Interstitial lung disease (ILD)	ILD was defined as the presence of pulmonary fibrosis on lung imaging, usually by high resolution computed tomography scan.
Pulmonary Arterial Hypertension (PAH)	PAH was defined as a mean pulmonary artery pressure of ≥25 mm Hg and a pulmonary capillary wedge pressure of ≤15 mm Hg on right sided heart catheterization.
Systemic Hypertension	Systolic BP ≥ 140 mmHg or diastolic BP ≥ 90 mmHg

Variable	Description
Home oxygen	For either severe PAH or severe ILD, ever
Gastrointestinal involvement	Defined as one or more of symptomatic or endoscopically proven oesophageal reflux, oesophageal dysmotility or oesophageal stricture, gastric antral vascular ectasia, or symptoms of faecal incontinence or small bowel involvement such as pseudo-obstruction with a positive response to antibiotics, or radiographically proven small bowel involvement either by barium studies or prolonged nuclear transit time.
Renal crisis	Defined as an abrupt onset of severe hypertension (systolic blood pressure (BP) ≥ 180 mmHg and/or diastolic BP ≥ 100 mmHg) without an alternate etiology, with or without microangiopathic anemia or decline in renal function
Renal Transplant or Renal involvement	Yes/No
Estimated glomerular filtration rate (eGFR)	Lowest ever
Cardiac involvement	Defined as the presence of either left ventricular systolic or diastolic dysfunction where no other cause was identified, or a conduction disturbance unexplained by other mechanisms, or a characteristic histological picture on endomyocardial biopsy.
Electrocardiogram (ECG) abnormalities	Ever
ECG Left bundle branch block (LBBB)	Yes/No or N/A
ECG Right bundle branch block (RBBB)	Yes/No or N/A
Echo pericardial effusion	Ever
Echo Left ventricular systolic	Abnormal ever
Echo, Lowest Left Ventricular Ejection Fraction	Lowest value Ever (%)
Echo sPAP (mmHG)	Highest Ever
Myocardial disease	Ever
Myositis	Defined as the presence of muscle weakness and/or muscle pain with an elevated serum creatinine kinase, or the presence of inflammatory muscle disease on a muscle biopsy.
Erythrocyte sedimentation (ESR) rate	Highest ever
Creatinine level	Highest ever collected annually
C-Reactive Protein	Highest ever
Serum Creatine kinase (CK)	Laboratory reported level (µL)
C3 and C4 complement levels	Below normal ever
Dry eyes	Ever
Dry mouth	Ever
Malignancies	Type and date of diagnosis of were recorded. Malignancies that pre-dated or post-dated the diagnosis of SSc were included. Where applicable, the diagnosis of malignancy required histological confirmation. Malignancies were categorized as solid organ, hematopoietic or skin (non-melanoma or melanoma). The 'other' category comprised neoplastic variants such as amyloidosis and pre-neoplastic conditions
Antinuclear antibody pattern by immunofluorescence on HEp-2 cells	ANA nucleolar pattern (AC - 8,9,10) ANA speckled pattern (AC – 2,4,5) ANA centromere pattern (AC – 3) ANA homogeneous pattern (AC – 1) Refer to the official website for the International Consensus on Antinuclear Antibody (ANA) Pattern (ICAP). http://www.anapatterns.org/
ASIG autoantibody testing at individual specialist centre laboratories.	Antibodies to extractable nuclear antigens (Orgentec ELISA, Mainz, Germany) including antibodies to Topo1, CENP, RNAP3 U1RNP, Jo-1, Ro52/Ro60, La, Sm and PM-Scl; antibodies to double-stranded DNA (Amerlex radioimmunoassay; Trinity Biotech, Bray, Ireland); anti-neutrophil cytoplasmic antibodies (Orgentec ELISA) including proteinase-3 or myeloperoxidase specificity; rheumatoid factor; antiphospholipid antibodies including anti-cardiolipin antibodies (Vital Diagnostics, Bella Vista, NSW, Australia); anti-b2 glycoprotein antibodies (Orgentec ELISA). Other commercially available diagnostic kits may have been used in the testing for AA at the various participating centres.

Autoantibody analysis

Sera were analysed 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 -80ºC until the time of testing. The assay was performed according to the manufacturer'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 (hUBF), 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 topoisomerase I (Topo1 formerly Scl-70) isolated from calf and rabbit thymus. Sera were analysed at a dilution of 1:101, and autoantibodies were detected using alkaline phosphatase–labelled antihuman IgG. The EuroLine flatbed scanner provides semi quantitative 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. Appropriate positive and negative controls were included in each run. All scores were then used in the principal component analysis. Separate studies have found good correlation between the signal intensity in the immunoblot assay and autoantibody titres/concentrations measured in alternative assays (81, 116, 144, 200).

Initial ASIG ANA Detection

The initial presence of ANA was determined by Australian pathology laboratories that participate in the National Association of Testing Authorities (Australia) accredited by the Royal College of Pathologists, Australia. The cut off value distinguishing a negative or positive value were all determined locally according to established principals (289). The presence of mitotic spindle or mitotic staining is most commonly reported as negative at local labs, however some may report it as positive staining.

U1RNP

Anti-U1RNP antibody is an important AA in the SSc spectrum and is not available on the EuroLine (Euroimmun) SSc immunoblot. Previous testing for U1RNP by independent laboratories from ASIG specialist centres had been completed. Following the SSc immunoblot, selected patient results were examined for the following reasons:

i. Speckled ANA.
ii. Negative results in the SSc line blot but with a previous U1RNP positive test result.
iii. Positive monospecific for TRIM21/Ro52 SSc blot result.
iv. Sufficient sera were available for testing.

These sera were then analysed using the EuroLine (Euroimmun) ANA Profile 5 (IgG) to ascertain if any were U1RNP positive to compare to the ASIG database. All samples were analysed in a single laboratory by a single operator (KAP). Serum aliquots were stored at -80ºC until the time of testing. The assay was performed according to the manufacturer's instructions. The kit contains 18 different antigens (IgG class), however the antigens of interest were RNP/Sm, native U1RNP purified by affinity chromatography from calf and rabbit thymus, RNP 70, -A, -C; recombinant U1RNP proteins. The corresponding cDNA is expressed in insect cells using a baculovirus vector or in E.coli (RNP 70). Sera were analysed at a dilution of 1:101, and autoantibodies were detected using alkaline phosphatase–labelled antihuman IgG. The EuroLine flatbed scanner provides semi quantitative 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. Appropriate positive and negative controls were included in each run. All positive scores were then used as supplementary individuals in the principal component analysis.

U1RNP positive patients were compared with all other patients in the ASCS cohort and also with patients within Cluster 4. However, in accord with the literature where U1RNP confers an 'overlap' variant, patients are analysed on an AA positive/negative status.

ANA and EuroLine Blot Negative

Where testing indicated a negative Immunoblot (SSc and ANA 5) result coupled with a negative ANA result by ASIG associated independent laboratories, further testing on these specific patient sera were performed. This supplementary testing was

initially by indirect immunofluorescence (IIF) on HEp-2 cells followed by extractable nuclear antibody tests using counter immunoelectrophoresis precipitation. Counter-immunoelectrophoresis (CIEP) was conducted using rabbit thymus extract and a human cell line K562 as the source of solubilized nuclear extract. AAs tested include Ro, La, RNP, Sm, Topo1, Jo1 PmScl and signal recognition protein. IIF pattern analysis did not indicate RNAP3 positivity. Appropriate positive and negative controls were included in each run. Results for IIF were considered negative on titres less than or equal to 1/80. CIEP results were considered negative if no precipitation was visible between the control negative antigen well and the antibody well. Both ANA and CIEP supplementary testing were carried out at the Flinders Medical Centre) SA Pathology Immunology Laboratory, which takes part in the National Association of Testing Authorities (Australia) accredited Royal College of Pathologists Australia Quality Assurance Program in Immunopathology on an annual basis.

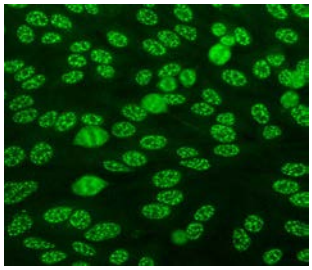


Figure 2-1: ANA Speckled Pattern

http://pathlabs.ribhyt.nhs/antinuclearantigen

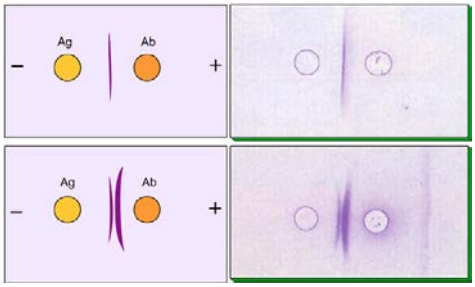


Figure 2-2: Counter-immuno-electrophoresis (CIEP) positive and negative Antibody/antigen interaction.

Statistical analysis

Following is a detailed explanation of the process undertaken and the terminology used in the analysis of data generated in this study.

Terminology

Eigenvectors and Eigenvalues

Eigenvalues are a specific measurement utilised in matrix equations to determine both the magnitude and direction (eigenvectors) of relationships between individual components. Eigenvectors and eigenvalues are therefore paired measures, every eigenvector has an eigenvalue. An eigenvalue is a quantifiable coefficient calculated to explain the variance in the data and the eigenvector determines the direction of the variance. Any factor with an eigenvalue ≥1 explains more variance than a single observed variable.

Horn's Parallel Analysis

A parallel analysis is a method based on the generation of random variables to determine the number of factors to retain in matrix analysis (290). Horn's parallel analysis compares the observed eigenvalues extracted from a correlation matrix to be analysed with those obtained from uncorrelated normal variables. A factor is considered significant if the associated eigenvalue is bigger than the mean of those obtained from the random uncorrelated data (291).

Analysis

Principal Component Analysis (PCA) on the ASIG patient database (n=505)

To enable the reduction of multi collinear data as a means of exploring underlying structures, the data were examined by PCA of the autoantibody scores as performed in the R Library Facto-MineR (292) . Essentially the PCA was the chosen means of analysis as this method identifies the fundamental elements of a dataset and quantifies their relationship to each other. Dimensions are calculated to organise data with the purpose of reducing to the least number of dimensions to explain the variance in the data.

Examination of the correlations between each autoantibody by the PCA dimensions revealed that only dimensions 1 and 2 captured both positive and negative relationships between different autoantibodies, and therefore, these two dimensions were subsequently used for hierarchical clustering of SSc patients into autoantibody-defined subgroups.

In Table 2-2, each dimension can be interpreted by examining the correlations with the autoantibody scores, and the strongest correlations are marked in bold. The number of dimensions (Dim) to retain in the PCA analysis was governed by Horn's parallel analysis to determine adjusted eigenvalues >1. Examination of the correlations between each autoantibody and the PCA dimensions reveals that only Dim1 and Dim2 captured both positive and negative relationships between different autoantibodies, and these two dimensions were subsequently used for hierarchical clustering of SSc patients.

Table 2-2: Entire ASCS PCA. Correlation between autoantibody score and each PCA dimension.

Autoantibody	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5
Ro52	0.24	0.15	0.05	0.44	0.44
Ku	-0.03	0.06	-0.50	**0.53**	-0.23
PM75	0.10	-0.08	**0.65**	0.33	-0.22
PM100	0.12	-0.05	**0.58**	0.31	-0.41
Th.To	0.00	-0.05	0.26	0.34	**0.70**
NOR90	0.04	0.26	0.16	-0.10	0.03
Fib	-0.12	0.07	-0.39	**0.61**	-0.19
RNAP11	**-0.62**	**0.69**	0.09	-0.07	-0.03
RNAP155	**-0.62**	**0.68**	0.08	-0.07	-0.02
CENPA	**0.89**	0.34	-0.08	-0.12	-0.06
CENPB	**0.89**	0.33	-0.05	-0.11	-0.06
Topo1	-0.33	**-0.71**	-0.03	-0.15	-0.05
PDGFR	0.03	-0.05	-0.09	-0.05	0.27
Unadjusted Eigenvalues	2.60	1.80	1.30	1.20	1.10
Adjusted Eigenvalues[1]	2.30	1.60	1.10	1.10	1.0
Variance %	19.6	13.6	9.9	9.5	8.2
Cumulative Variance %	19.6	33.3	43.2	52.7	60.9

[1]Adjustment by Horn's parallel analysis

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 analysed using IBM SPSS Statistics software version 22.0. Significance was set at $p \leq 0.05$.

The Cluster 4 PCA

The second PCA used IBM SPSS Version 22.0 (Armonk, NY) software as R was not available. A detailed description of the PCA process using SPSS follows.

Kaiser-Meyer-Olkin (KMO) and Bartlett's Measure of Sampling Adequacy

The KMO and Bartlett's test is a statistic that indicates the proportion of variance in the data that might be caused by underlying factors. The KMO and Bartlett's Test (p=0.006) established that 46.6% of the data were influenced by underlying factors.

Scree Plot

The scree plot utilises eigenvalues associated in descending order against components. Scree plots assist in the assessment of those components that explain the most variability in the data. Eigenvalues ranged from 1.679 in component 1 to 1.442 in component 2 to 1.143 in component 3.The decision where to 'cut' the data was at the third component with an eigenvalue of 1.143 (adjusted by Horn's parallel analysis). The scree plot for this PCA is visualised in Figure 2-3.

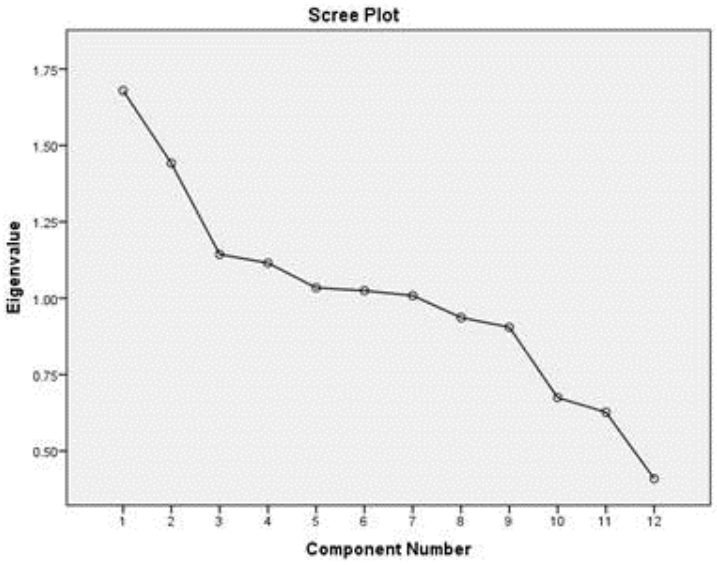


Figure 2-3: Cluster 4 Scree plot associated with the Supplementary PCA of Cluster 4

Communalities

Extraction communalities are estimates of the variance in each variable that is accounted for by the components. High values (>.500), represent the magnitude each component contributes to the overall variance and values that fall below .500 do not contribute significantly to the overall variance. Figure 2-4 demonstrates the variance accounted for in each variable after three extractions as determined by the scree plot and adjusted Eigenvalues. The highest values for variance were Topo1 (69.4%), CENP B (67%), PM75 (67.9%), TRIM21/Ro52 (66.7%), PM100 (54.6%), and to a lesser extent Th/To (41.9%).

Table 2-2: Extraction Communalties for Cluster 4 PCA

Communalities

	Initial	Extraction
TRIM21	1.000	.667
Ku	1.000	.059
PM75	1.000	.679
PM100	1.000	.546
Th/To	1.000	.419
NOR90	1.000	.127
Fib	1.000	.026
RNAP11	1.000	.071
RNAP155	1.000	.251
CENP A	1.000	.055
CENP B	1.000	.670
Topo 1	1.000	.694

Extraction Method: Principal Component Analysis.

From the above analyses, a second PCA was generated showing both positive and negative relationships across three components. A pattern matrix (Table 2-4), contains loading strengths (-1 to +1), demonstrating correlations between components. A factor loading of at least ±.320 is considered to be statistically meaningful to the understanding of relationships between variables, both in and between dimensions (293). The underlying influence in Component 1 is CENP B and Topo1 (highlighted in orange). The underlying influence in Component 2 is PmScl (highlighted in blue). In Component 3, TRIM21/Ro52 and Th/To have a significant contribution (highlighted in yellow). A subsequent hierarchical clustering of autoantibodies identified in the PCA, showed the AAs with the most influence in Cluster 4 were TRIM21/Ro52, PmScl, Topo1, CENPB and Th/To (Figure 2-4)

Table 2-4: Pattern matrix correlation between autoantibody score and each PCA component.

Autoantibody	Component 1	2	3
CENP B	.817	.036	.034
Topo 1	.816	-.208	-.019
RNAP155	.338	.173	-.275
PM75	.151	.808	.242
PM100	-.116	.735	.000
Ku	-.112	-.209	.007
CENP A	.130	-.199	.013
Fib	.041	-.130	.074
TRIM21	.369	.056	.758
Th/To	-.039	.272	.629
NOR90	-.087	-.167	.271
RNAP11	-.096	-.068	.222

Extraction Method: Principal Component Analysis.
Rotation Method: Oblimin with Kaiser Normalisations.

a Rotation converged in 8 iterations.
Colours represent the strongest AA relationships in the component numbered 1, 2, or 3.

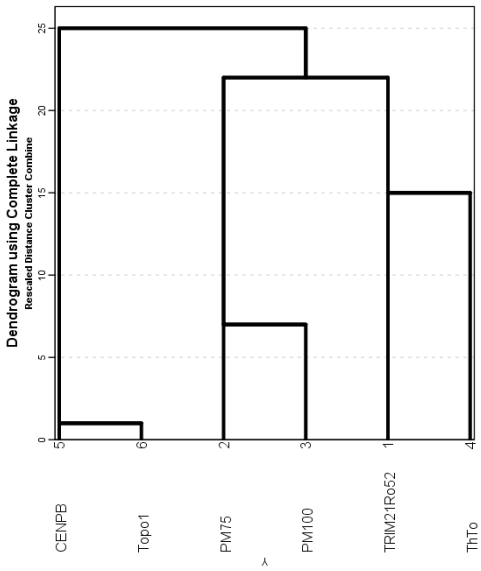


Figure 2-4: Hierarchical clustering of Cluster 4 autoantibodies

Of interest, there was the demonstrated association of TRIM21/Ro52 with each of the other antibodies. This can be seen in Chapter 4, Table 4-1 (p.95) where TRIM21/Ro52 is the most frequently co-expressed AA.

The first PCA on the entire data set demonstrated strong associations. The second PCA in Cluster 4 revealed that the association between the AAs were much weaker. This is logical as Cluster 4 patients were those that do not have a strong relationship with one of the three primary AAs and were more clinically heterogeneous in their AA subgroups.

Clinical associations with Autoantibodies

Where numbers permitted, statistical analysis was used to explore any clinical associations with the SSc associated AAs. Statistical analyses were undertaken to explore the clinical features of any patients testing positive to one of the three primary AA. To limit bias, only patients monospecific for the less specific or rarer disease associated AA were tested. Fisher's Exact method was used for discreet nominal variables. Continuous variables were assessed with ANOVA, using Levene's test for homogeneity of variances. To protect against a Type 1 error, if homogeneity of variances were violated, a Mann-Whitney U test was applied and this is annotated where undertaken. Results $p \leq 0.05$ are considered statistically significant. In some instances trends were reported where they were found to be significant in other cohorts and are reported where $p<0.09$.

ANA, ENA and EuroLine blot negative

Data were analysed using IBM SPSS Statistics Version 22.0 (Armonk, NY). Clinical manifestations were dependent variables and ANA status was the independent variable. Continuous variables were assessed with ANOVA. Due to the small and unequal sample size of the AA negative subgroup compared to the AA positive subgroup, bootstrapping, the Levene's test for equality of variances, Mann Whitney U test and Kruskal-Wallis tests were used. For categorical variables a Chi Square independence test, bootstrapping and Fishers Exact methods were employed, again to compare AA negative and AA positive groups. Significance was set at $p \leq 0.05$.

Comparison of laboratory methods – LIA, IIF and IP

Cohen's Kappa statistic (kappa or κ) is frequently used to test interrater reliability on qualitative data. The importance of interrater reliability is that it represents the extent to which the data collected in the study are correct representations of the variables measured (294).The kappa statistic ranges between −1 to +1. A rating of 0 represents the amount of agreement that can be expected from random chance, and 1 represents perfect agreement between the methods of rating. While kappa values below 0 are possible, they are unlikely in practice (294). The kappa statistic is a standardized value and thus is interpreted the same across multiple studies (294).

Various opinions are published on what is considered an acceptable κ score. Table 2-4 describes the various strength of agreement of the κ statistic based on the assessment by Landis and Koch (295), Altman (296) and Fleiss et al (297).

Table 2-4: The Kappa statistic and strength of agreement, citing literature

Author	Kappa Statistic (κ)	Strength of Agreement
Landis and Koch	0.81 – 1.00	Excellent
	0.61 – 0.80	Substantial
	0.41 - 0.60	Moderate
	0.21 – 0.40	Fair
	0.01 – 0.20	Slight
	<0.00	Poor
Altman	0.81 – 1.00	Very good
	0.61 – 0.80	Good
	0.41 – 0.60	Moderate
	0.21 – 0.40	Fair
	< 0.20	Poor
Fleiss et al	0.75 – 1.00	Very Good
	0.41 – 0.75	Fair to Good
	<0.40	Poor

CHAPTER 3
INTERPRETATION OF AN EXTENDED AUTOANTIBODY PROFILE IN A WELL CHARACTERISED AUSTRALIAN SYSTEMIC SCLEROSIS (SCLERODERMA) COHORT UTILISING PRINCIPAL COMPONENT ANALYSIS.

Introduction

Systemic sclerosis is a heterogeneous disorder with well documented genetic (298-301) and geographic variation (302). 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 sub-classification in a condition that fluctuates with time. In 1980, the ACR 'Preliminary criteria for the classification of systemic sclerosis' (SSc) (286) were published. Since then there has been continuing discussion about whether this classification tool should be expanded to include all patients with variant subsets (42) who fall within the Scleroderma Spectrum Disorders (53, 303). Initial validation studies of 2013 ACR/EULAR classification criteria for SSc (43) show improved accuracy but do not encompass sub- classification nor do they provide prognostic information. Improved biomarkers for the sub classification of SSc subsets are sorely needed.

Serum AAs are found in up to 95% of patients with SSc (65) and carry greater weight in the newly proposed ACR/EULAR classification system. CENP, Topo1 and RNAP3 closely reflect patterns of organ involvement and disease progression (65), and with rare exceptions they appear to be mutually exclusive at this level of AA analysis. Other serum AAs are known to be associated with SSc (22, 76, 304) but their clinical associations and frequencies are less well defined. Until recently, many of these rarer AAs were only available in the research setting and required labour intensive laboratory analyses. The advent of new, less labour-intensive technologies, such as the line immunoassay (LIA) means multiple AAs, including rarer AAs, can now be tested in routine clinical practice. The clinical significance of these rarer AAs remains uncertain, as does the significance of multiple AA positivity. We report on the clinical and serological associations of ten AAs in a large, well characterised multi-centre study of SSc patients utilising LIA. The results demonstrate that the use of an extended AA provides useful prognostic information at the time of diagnosis and confirms the utility of AAs in the SSc spectrum of disorders. Autoantibody profiling will, we predict, have added potential in allowing sub-classification and stratification of patients into clinical trials.

Results

Demographical, clinical and serological characteristics are presented in Table 3-1. At the time of entry into the ASCS, patients in this cohort were judged to have SSc by the 1980 ACR criteria, Medsger criteria, or by expert opinion. At the time of data censorship, the ACR/EULAR 2013 data were not yet published, however retrospective analysis showed that 498/505 (98%) of our patients fulfilled the revised criteria.

Table 3-1: Demographic, clinical, and serologic characteristics of the 505 SSc patients from the ASCS*

Characteristic	N (%) or mean ± SD	Total Number Patients
Patients		**505**
Female	443 (87.7)	
Male	62 (12.3)	
Female to male ratio	7:1	
Ethnicity		**505**
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		**505**
Diffuse	135 (26.7)	
Limited	370 (73.3)	
Fulfilled 2013 EULAR/ACR criteria	498 (98)	
Fulfilled 1980 ACR criteria	454 (89.9)	
Age (years)	**63.31 ± 12.31**	**505**
Age Onset Raynaud's (years)	41.4 ±15.77	490/505
Age onset symptoms (non Raynaud's) (years)	46.01 ± 14.06	498/505
Disease duration (years)	11.96 ±9.97	498/505
ANA IIF Positive	476 (94.3)	
ANA Nucleolar	125 (24.8)	
Speckled	138 (27.3)	
Centromere	212 (42)	
Homogeneous	113 (22.4)	

ANA – antinuclear antibody * 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.

Autoantibody Analysis

Frequency and Combination

Counts of individual AAs and their expression either monospecifically or the number of times they appear with other AAs can be seen in Table 3-2 and Figure 3-1. The percentages of AA frequencies are summarised as follows: 225 (45%) had a monospecific autoantibody while 165 (33%), 49 (9%) and 10 (2%) were positive for two, three or more AA respectively. The remaining 56 patients (11%) were negative for all AA by immunoblot testing (Figure 3-2).

The majority of patients were positive for one of three major AAs; CENP, Topo1 or RNAP3 and co-expression of AAs were common (Table 3-2). TRIM21/Ro52 was the most frequent autoantibody occurring in combination with other AAs and originally only 19 (3.76%) patients were monospecific for TRIM21/Ro52. Subsequent testing revealed that 5 patients who were TRIM21/Ro52 monospecific patients were also positive for U1RNP. Topo1 was the most frequent monospecific AA and PDGFR had the lowest monospecific AA expression. Co-expression of CENP and Topo1 occurred rarely (15/505).

Table 3-2: Autoantibody counts and combinations in ASCS patients, n=505.

Autoantibody	TRIM21/Ro52	Ku	Pm-Scl	Th/To	NOR90	Fibrillarin	RNAP3	CENP	Topo 1	PDGFR
TRIM21/Ro52	14	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
NOR90					1	0	5	11	1	0
Fibrillarin						1	2	0	1	0
RNAP3							28	13	3	0
CENP								86	15	1
Topo 1									68	1
PDGFR										
Monospecific AA[1] (%)	14 (8)	3 (21)	11 (12)	3 (20)	1 (5)	1 (17)	28 (35)	86 (38)	68 (61)	0
Total AA [2]	178	14	89	15	19	6	81	228	112	2

1 Monospecific expression of AA without any other AA co-expression 2 Total numbers of patients with expression of each AA. Due to multiple co-expression (2 or more AA), these numbers are less than the sum of the AA. (%) monospecific

PRGFR- platelet derived growth factor receptor, NOR90 - 90-kDa nucleolus organizer region, Pm-Scl-polymyositis/scleroderma (exosome), TRIM21/Ro52 - tripartite motif containing 21, RNAP3 - RNA Polymerase III, Topo1 -Topoisomerase1, CENP- Centromere protein A and B.

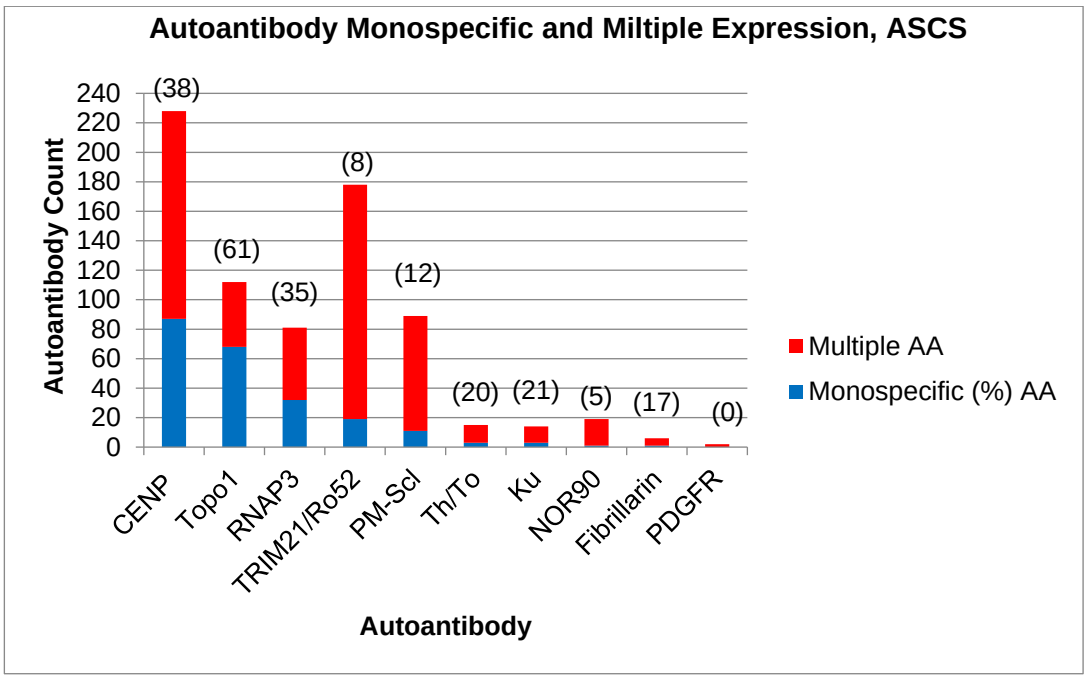


Figure 3-1: Individual autoantibody counts showing singular expression and multiple expressions in ASCS patients, n=505. Autoantigens defined as follows: PDGFR- platelet derived growth factor receptor, NOR90 - 90-kDa nucleolus organizer region, Pm-Scl- polymyositis/scleroderma (exosome), TRIM21/Ro52 -tripartite motif containing 21, RNAP3 - RNA Polymerase III, Topo1 – Topoisomerase1, CENP – Centromere protein A and B. (%) monospecific labelled at the top of each bar.

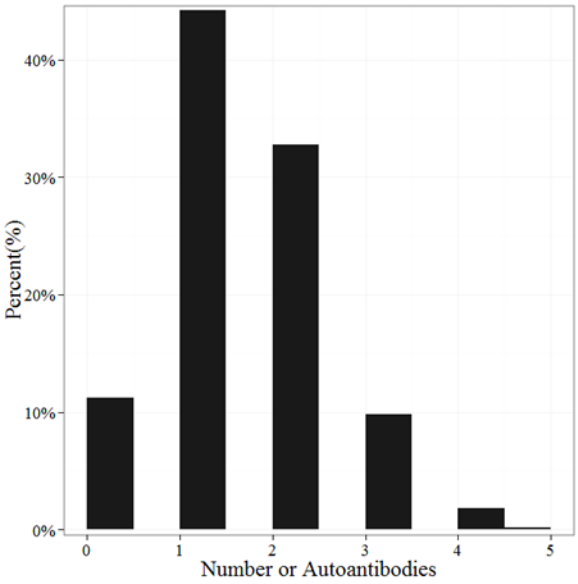


Figure 3-2: Percentage of patients with co expression of AA in ASCS, n=505

Cluster Analysis

Strong negative associations between the 3 major autoantibodies are evident (Figure 3A) with the PCA analysis of AAs revealing 5 major clusters (Figure 3B). Clustering was determined by the presence of one of the 3 major AAs and one further group that was positive for one or more of the other AAs tested.

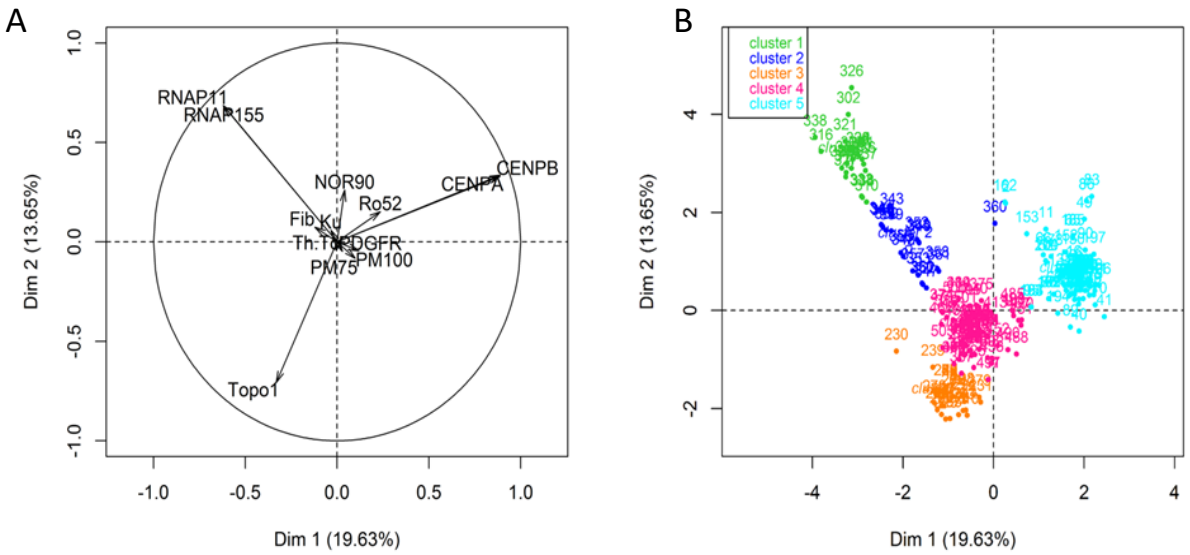


Figure3-3: Principal components analysis and hierarchical clustering of immunoblot assay autoantibody scores (range 0–3) in 505 patients with systemic sclerosis. 3A, Correlation circle plot of the first 2 dimensions (Dim 1 and Dim 2) of the principal components analysis, which accounted for 51.4% of the total variance. This plot illustrates strong correlations between RNA polymerase III epitope 11 (RNAP11) and RNAP3 epitope 155 as well as between CENP A and B. The mutually exclusive relationships between RNAP3, 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. 3B, Hierarchical clustering of the first 2 dimensions by principal components analysis, which resulted in 5 autoantibody clusters: RNAP3 strong positivity (39 patients [8%]; Cluster 1), RNAP3 weak positivity (25 patients [5%]; Cluster 2), Topo1 (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.

Heat map of Immunoblot scores

The association between the presence and staining intensity of specific AAs compared with the dichotomised classification of lcSSc and dcSSc and the pattern of organisation of the Clusters are demonstrated in the colour coded heat map (Figure 3-4).

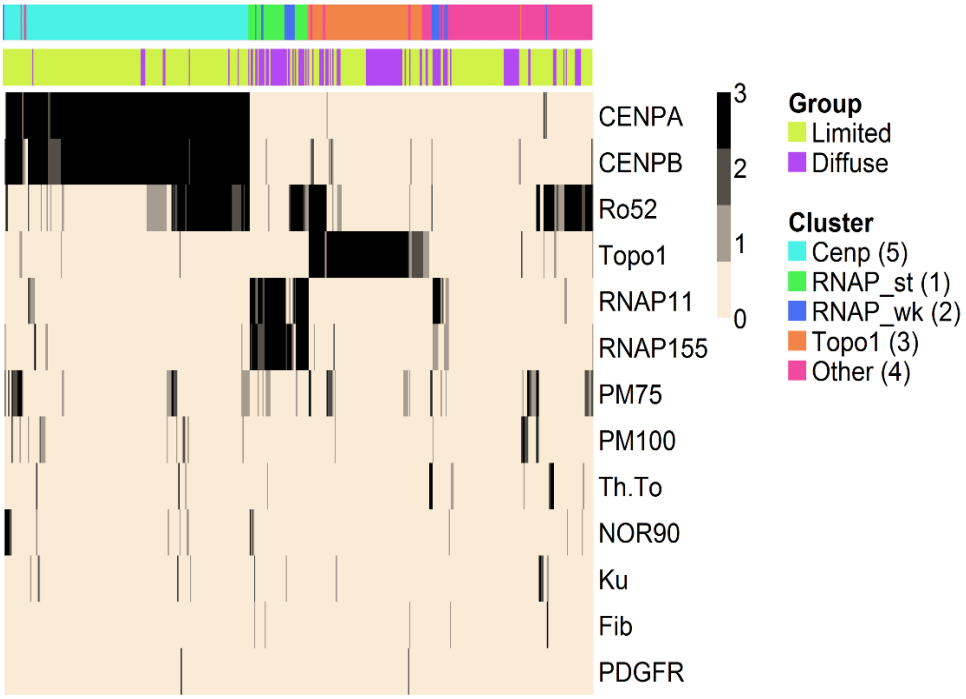


Figure 3-4: Heat map of the immunoblot assay autoantibody scores in 505 patients with systemic sclerosis (SSc). Individual patients are represented by the columns, and individual autoantibodies are represented by the rows. The annotation bars at the top designate the autoantibody cluster allocation for each patient (as described in Figure 3B) and the limited or diffuse classification of each patient's SSc. See Figure 3-3, p81 for other definitions.

Clinical associations of groups identified by Cluster analysis

The five SSc clusters identified by PCA were assessed with regards to their clinical characteristics (Table 3-3)

Cluster CENP revealed many features consistent with LSSc. Clusters associated with RNAP3 were statistically separated between 'strong positive' and 'weak positive'. Both Cluster RNAP3 'strong' and Cluster RNAP3 'weak' demonstrated multiple features of dcSSc as did Cluster Topo1. Cluster 'Other' was inversely associated with the presence of Raynaud's phenomenon, telangiectasia and joint contractures and was positively associated with male gender, a history of smoking and malignancy.

Table 3-3: Clinical associations of the 5 AA clusters

Demographic/Clinical Variable	All SSc	Cluster CENP n = 206	Cluster RNAP3 strong n = 39	Cluster RNAP3 weak n = 25	Cluster Topo1 n = 93	Cluster Other n = 142	Global p-value
Classification (Diffuse): %	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: %	95%	97%	97%	92%	97%	90%§	0.037¶
Telangiectasia: %	90%	94%§	92%	100%	87%	84%§	0.007¶
Smoking History: %	47%	47%	41%	48%	37%‡	56%‡	0.064
Malignancy (%)	13%	8%	16%	8%	12%	19%§	0.039¶
Gender (Males): %	12%	5%†	10%	4%	18%	20%†	< 0.001
Onset Age: mean	46	48‡	47	46	43‡	45	0.13
PAH: %	12%	17%§	13%	12%	4%§	10%	0.031¶
Calcinosis: %	43%	49%‡	53%	32%	32%‡	40%	0.039
Reflux oesophagitis: %	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¶,#
Oesophageal dysmotility: %	12%	13%	0%§	16%	8%	15%	0.053¶,#
Renal Transplant: %	1%	0%	0%	8%‡	0%	1%	0.006¶
ILD: %	33%	15%†	36%	32%	74%†	33%	< 0.001

* The systemic sclerosis (SSc) patients were allocated objectively into autoantibody clusters (see Figure 3B, p.81), and analysis was performed using the v test (see p.71), followed by Fisher's 2-sided exact test for rarer outcomes. Values are the percentage, except for age at onset, which is the mean. RNAP3 =RNA polymerase III; Topo I = topoisomerase I; GAVE = gastric antral vascular ectasia. † $p < 0.001$ versus the overall mean, as determined by v test.‡ $p < 0.05$ versus the overall mean, as determined by v test.§ $p < 0.01$ versus the overall mean, as determined by v test.¶ Significant association confirmed by Fisher's 2-sided exact test.# RNAP3 strong versus RNAP3 weak significantly different by Fisher's 2-sided exact test.

Division of Cluster 2 RNAP3 'strong' and Cluster 3 RNAP3 'weak'

Cluster RNAP3 'weak' has a weaker reactivity with RNAP3, either as a result of weaker scores or because it was only reactive to one epitope. The possible difference between the two clusters lies with the risk of GAVE and its association with RNAP3 'strong' only and oesophageal dysmotility, for which RNAP3 'strong' is less likely.

There was a trend for shorter disease duration for Cluster RNAP3 'strong' compared to Cluster RNAP3 'weak' (Figure 3-4). Modified Rodnan Skin Scores can be seen in Figure 3-5.

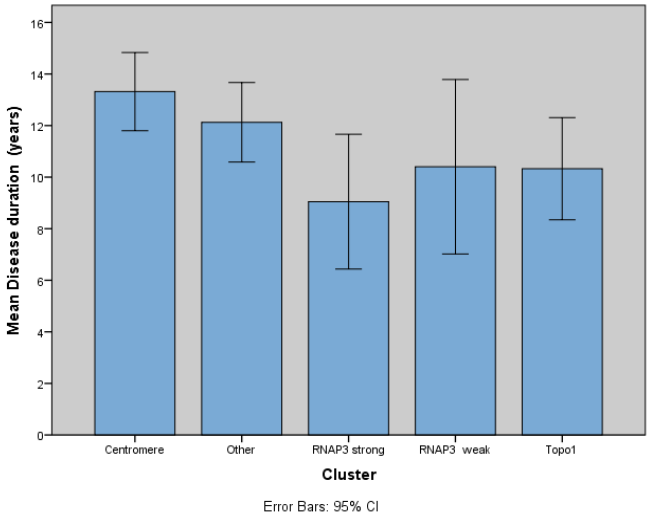


Figure 3-4: Mean disease duration (years) in ASCS by cluster, n=505

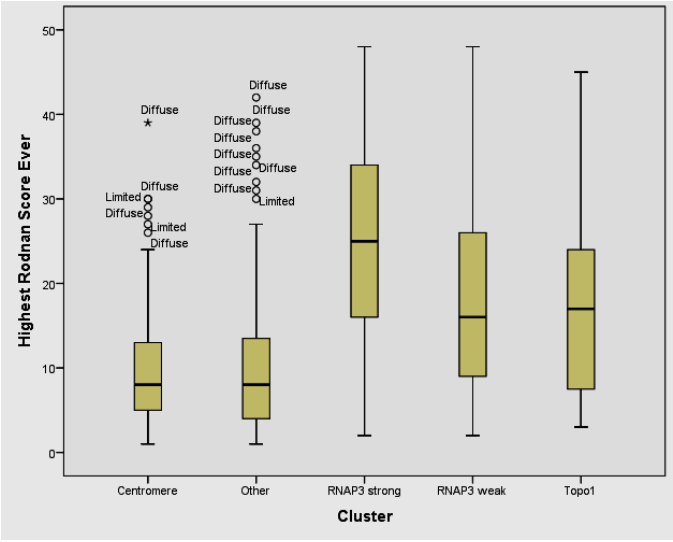


Figure 3-5: Modified Rodnan Skin Scores for each cluster. ºDenotes outliers and * denotes extreme outliers.

Other SSc associated autoantibodies

A univariate analysis was performed to look for clinical associations in the presence of TRIM21/Ro52, NOR 90, PmScl 75, PmScl100, ThTo, Ku, fibrillarin and PDGFR. Statistically significant associations, or those approaching statistical significance, are summarised in Table 3-4. Given the low numbers of patients with Th/To (15/505), Ku (14/505), Fibrillarin (6/505), NOR 90 (19/505) and PDGFR (2/505), we report any associations with caution, recognising 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 oesophagitis (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 towards the presence of GAVE (p=0.071). No significant associations were observed for NOR90 and PDGFR.

Table 3-4. 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.

TRIM21/Ro52 and PmScl75/100 were the most common of the other SSc associated AA with 35.3% and 17.6% of patients testing positive for these AAs respectively. While their presence did not contribute to sub-classification of patients, the clinical associations for TRIM21/Ro52 and PmScl75/100 are of interest and are summarised in Table 3-4. The presence of telangiectasia was strongly associated with TRIM21/Ro52 ($p = 0.01$) while adjusted data show a trend towards the presence of dry eyes, PAH and calcinosis. There was no evidence of an association with ILD in this cohort ($p=0.30$).

PmScl75/100 was associated with a history of digital ulcers ($p=0.032$) and patients expressing this AA show a trend towards the presence of lSSc ($p=0.052$). PmScl75/100 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 TRIM21/Ro52 and PMScl75/100 were more commonly seen in the presence of CENP.

Discussion

A scoring system that identifies both the presence and intensity of AA staining reduces bias from multiple AA positivity and confounding from false positive results. The presence of AAs do not change over time (305, 306) although staining intensity may vary. Others have found AAs with the highest titre are the most strongly associated with the clinical phenotype (22) and may be more reliable than clinical skin scores which are subject to inter-observer variability (307, 308). Our findings extend these observations by demonstrating that this remains true even when possible modulation of disease by the presence of multiple positive AAs are considered.

In the presence of multiple AAs, CENP, Topo1 and RNAP3 remain the most common SSc specific AAs in this cohort and have strong, clinical associations. While co-expression of any of these three major AAs remains rare (Table 3-2), co-expression with other AAs are frequent in our cohort and others (22, 81, 304). PCA followed by hierarchical clustering provides a novel means to interpret these complex associations by considering only the dominant AA and fosters uniformity within each AA cluster.

Cluster CENP (41%) positive patients were predominantly female and had significant associations with calcinosis, reflux oesophagitis, sicca, telangiectasia, anal incontinence and PAH. ILD, joint contractures, tendon friction rubs and synovitis were reduced compared with other clusters. The clinical associations of CENP remain across geographic locations and ethnic groups (110, 112, 305, 309) and provide a universal prognostic marker.

RNAP3 was associated with two different AA clusters, depending on the intensity of RNAP3 staining (Cluster RNAP3 ‘strong’ (8%) and Cluster RNAP3 ‘weak’ (5%)). RNAP3 titres change over time (310), and in our analysis there was a trend towards increased titres in those with earlier disease. Therefore, Clusters RNAP3 ‘strong’ and Cluster RNAP3 ‘weak’ may represent different temporal stages of SSc disease. The trend towards Cluster RNAP3 ‘strong’ representing earlier disease and an association with scleroderma renal crisis (SRC) and GAVE is consistent with other studies where both SRC and GAVE are more likely to occur in early disease (35, 170, 311). Cluster RNAP3 ‘strong’ appeared less likely to have oesophageal dysmotility which is an intriguing finding, perhaps the invasive nature of assessing and confirming oesophageal dysmotility may see it under-reported in our cohort as other studies have reported early involvement in the gastrointestinal tract, even without symptoms (312, 313).

Prevalence rates for RNAP3 vary according to ethnicity and geographic location. The frequency of RNAP3 in this cohort lies somewhere between rates identified in other international cohorts (81, 117, 122, 166, 304, 314-317). Cluster RNAP3 'strong' had the highest mRSS while Cluster RNAP3 'weak' had the second highest mRSS, equalling that for Cluster Topo1.The clinical associations of high mRSS, SRC and now GAVE are consistent globally even though prevalence rates for this AA vary (126, 316).

Previous studies have found an association between the diagnosis of SSc and malignancy with RNAP3 (161, 173, 318), while our study did not. One possible reason for this finding is that malignancy in this study was defined as either haematological or solid tumour with non-melanoma skin cancers excluded. Non-melanoma skin cancer is by far the most common cancer diagnosed in Australia (319).

The remainder of those patients who had a clinical association with dcSSc were captured in Cluster Topo1 (18%). Topo1 frequencies were consistent with other international registries with similar ethnicity but varying geographic locations and it should be noted that frequencies of this AA vary with ethnicity (22, 81, 110, 112, 117, 118, 120, 122, 166, 304, 314, 315, 317, 320). Significant clinical associations in this cohort include high mRSS, ILD, joint contractures and digital ulcers.

Co-expression between the three primary AAs was only rarely observed in this cohort (Table 3-2), 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 (22, 317). It would be intriguing to investigate this group further, to identify if they share any common 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 others who have published using this technology, Mierau et al (81) from the German Network for Systemic Scleroderma Registry, used a line immunoassay (Euroimmun LIA) and found that co-expression of any combination of CENP, RNAP3 and Topo1 was rare, while co-expression of additional non-specific AA were common. In addition, Graf et al (22), used an identical assay to our study and also reported co-expression of both disease specific and non-specific AAs. They concluded that the clinical phenotype was most strongly represented by characteristics generally associated with the dominant AA and these findings are replicated in our study.

Cluster 4 ‘Other’ (28%) captures the remaining patients in our cohort. This cluster includes patients who (i) do not express CENP, Topo1 or RNAP3, (ii) may have a low score for one of the primary SSc AAs coupled with one or more of the SSc associated AAs or (iii) they may have either monospecificity or multispecificity for the SSc associated AAs.

Positive associations in Cluster 4 ‘Other’ include male gender, a history of malignancy and smoking. Negative associations include telangiectasia, Raynaud’s phenomenon and joint contractures. The clinical associations of this group are intriguing and warrant further investigation. U1RNP was not available on the SSc immunoblot; however in separate testing of all patients for this AA, all those classified with MCTD mapped to Cluster ‘Other’ (data not shown). If U1RNP had been included on the immunoblot, it may have formed a separate cluster with its own specific clinical associations. It is likely that Cluster 4 ‘Other’ identifies more than one disease group.

The rarer SSc associated AAs were also assessed in this study but they were not found to significantly contribute to sub-classification with PCA. Only a small number of patients tested positive for NOR90, PDGFR, Ku, Fibrillarin and ThTo (to hPOP1 epitope only) and it is likely that large multinational studies will be necessary to determine if they have significant clinical associations.

Where statistical power has permitted, both TRIM21/Ro52 and Pm-Scl were found to have more meaningful results. TRIM21/Ro52 is a common AA and its presence in SSc has been reported elsewhere, with varying clinical associations (22, 81, 304, 321). Of particular significance with regards to disease prognostication have been reports of associations with overlap disease and ILD (304). In this cohort, the presence of TRIM21/Ro52 was associated with telangiectasia and Raynaud’s phenomenon, with a trend towards the presence of dry eyes, calcinosis and limited disease. These clinical features are consistent with others who have reported associations with limited disease (22, 81) and sicca features (81). The trend towards PAH is intriguing and has not been reported elsewhere although direct comparison between the groups is difficult as right heart catheterisation is not readily available in all cohorts. The CSRG registry used a surrogate marker obtained from echocardiogram (pulmonary arterial hypertension) while Mierau et al did not provide a detailed definition of pulmonary HT (81). Differing methods used to detect TRIM21/Ro52 may also account for variations between cohorts. However, it is possible that the use of right heart catheter 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 (304), we did not find an association between TRIM21/Ro52 and ILD although the definition for ILD between groups varied significantly. In our cohort, ILD was defined on the basis of radiological findings, while Hudson et al used a recently published algorithm for ILD which included patients with typical clinical findings in the absence of radiological findings.

The presence of PmScl75/100 in SSc has been associated with limited and overlap disease, skeletal muscle disease, calcinosis, improved survival (81, 223, 227) and more recently, improved prognosis in ILD (322). Techniques used to detect these AA varied considerably between studies and so once again, this must be taken into account when interpreting findings between cohorts. In this cohort, PmScl75/100 was associated with digital ulcers and there was a trend towards an increased presence in limited disease. No significant associations were observed with a history of myositis or elevated CK (unpublished data).

Autoantibody classification of scleroderma versus traditional classification

Traditionally, SSc is classified according to the extent of skin fibrosis. Limited cutaneous SSc and dcSSc have well-established differences in their presentation, AAs and outcomes. However, the dermatological changes are a dynamic process and hence early labelling of a patient with limited disease may need to be modified later during the disease course.

The difficulties in diagnosis and sub classification of SSc based on skin involvement have direct relevance in the clinical setting. Virendrakumar Bhavsar et al. recently reported two cases of RNAP3 associated **SRC** occurring in the absence of initial skin involvement (323) presenting a major diagnostic challenge. Furthermore, Cottrell et al concluded that dichotomous classification based on skin involvement may result in misclassifying an intermediate group of patients who exhibit a unique AA profile, disease course and clinical outcome (283).

In contrast AAs are a consistent feature of the disease and it is rare for an AA to disappear, although fluctuations in antibody titre 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 AA. It seems logical therefore, to propose disease stratification based on AAs would be simpler to apply and be more consistent irrespective of stage of disease than the traditional classification system based on the extent of skin involvement. Furthermore, this cross–sectional study demonstrates strong disease associations with the AAs and may have important applications in

enriching studies of new therapies for certain subset(s) and identifying subjects for targeted therapies.

Strengths and Limitations

There were minimal missing data enabling a comprehensive statistical analysis of the results of the AA testing with the clinical and demographical variables. Limitations include the small number of patients with rarer AAs. While the clinical associations with the AAs in this study were similar to other published work, it is important to recognize that there may be different associations depending on how the AAs are identified by various platforms currently in use. Assays targeting a specific peptide AA sequence do not necessarily reflect the clinical associations of AA identified using more labour intensive assays that identify the whole protein, or indeed functional assays. The sensitivity often reported in LIA 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-characterised SSc registries.

Final comments and conclusion

PCA provides a novel means to identify the presence and intensity of scleroderma associated AAs and to reduce confounding when more than one AA is detected in patient sera. We have shown that sub-classification based only on the presence of AAs reveals clinically meaningful disease associations. The dominant AA is the one that most accurately reflects disease associations, at least when CENP, Topo1 and RNAP3 are considered. Two separate clusters of RNAP3 were identified with intriguing differences in clinical associations. We propose that this may relate to the well-documented fluctuations in RNAP3 over time, with higher titres being observed earlier in the disease course. Further prospective studies will provide further clarification in this regard. In our cohort, those who did not map to one of the 4 major AA associated clusters had intriguing clinical associations. They are likely to represent more than one disease group and warrant further investigation.

We conclude that the use of specific and associated SSc AAs provides more meaningful classification of subsets than the currently utilized limited, diffuse and overlap subdivisions. We also propose that using AA profiling for sub classification and stratification will improve disease management and prognostication and the identification of patients for clinical trials.

CHAPTER 4
CLUSTER 4, 'OTHER', EXPLORING THE CLINICAL UTILITY OF SYSTEMIC SCLEROSIS PRIMARY AND ASSOCIATED AUTOANTIBODIES

Introduction

In Chapter 3 an examination was conducted on the clinical and serological features of SSc-specific and associated autoantibodies (AA) and the stratification of these AAs into discrete 'Clusters' directed by a defining or primary SSc AA. The term 'primary' AA is used throughout this chapter when referring to Topoisomerase1 (Topo1), Centromere A and/or B (CENP A and/or CENP B) and RNA Polymerase III (RNAP3) as these AAs are considered to be specific for SSc (43). The clusters determined by PCA considered both the type of AA and the intensity of staining measured by the Euroimmun line immunoblot assay (LIA).

Among 505 patients with available sera, we found that the majority (72%) could be grouped according to an AA profile that was largely determined by the presence of one of the primary SSc associated AA. Furthermore, when these groupings were assessed according to their clinical characteristics, CENP A/B was found to associate with clinical features known to occur in lcSSc and RNAP3 and Topo1 with clinical features seen in dcSSc, *independent* of any clinical assessment. These findings suggest that these AAs may be used as sole criterions to sub classify SSc, a finding that has particular relevance when considering early disease prognostication and assessing patients for their suitability in clinical trials.

There remained 28% of patients who were not clearly linked to one of the primary AA groupings identified using cluster analysis (i.e. Clusters 1, 2, 3, & 5). For the most part, these patients did not have one of the three primary SSc AAs or if they did, it was of a lower staining intensity, or was present in combination with other SSc associated AAs. U1RNP testing was not available on initial immunoblot analysis and may have provided further explanation for patients without a detectable specific SSc associated AA. We were particularly interested to examine this cohort further as patients presenting with mixed or less specific AA profiles may offer a greater diagnostic and prognostic challenge to the clinician. The total number of patients in Cluster 4 is relatively small (n=142) meaning that care must be taken with analysis to limit bias. Therefore we decided to review AA groupings in the Cluster 4 cohort as a whole before evaluating any unique or defining disease features according to the dominant AA profile. Where numbers permitted, statistical analysis was used to

assess results.

Specific aims for this study were;

i. To further characterise the specific AA profile/s identified by the Euroimmun Line Immunoassay (LIA) and to identify any unique features between patients within this Cluster with a less homogeneous AA profile and to determine if variance in Cluster 4 is due to identifiable, underlying factors.
ii. To assess any unique clinical features associated with AA profiling in this cohort among Cluster 4 patients and among the SSc cohort as a whole.
iii. To explore if patients in Cluster 4, who were positive for one or more of the three primary AAs, differed clinically to patients positive for these AA but assigned by PCA to other Clusters (discussed in Chapter 3).
iv. To explore the clinical characteristics of Cluster 4 patients who are positive for both CENP and Topo1.
v. To assess this well characterised SSc cohort (see Chapter 2, pp. 64-66), for U1RNP and to compare clinical features among those patients whose sera contain U1RNP with a randomly selected cohort of patients with MCTD who are enrolled in the ASIG database

An initial analysis was carried out on patients that have the three primary AAs; CENP, Topo1 and RNAP3. Following that, analyses on the AAs that feature strongly in this Cluster; TRIM21/Ro52, PmScl and U1RNP were performed.

Methods

Please refer to Chapter 2, Methodology, (pp.64-66), for details regarding the patient population, Scleroderma Euroline assay and U1RNP testing. We had additional AA sera and clinical data on 21 patients identified to have MCTD in the ASIG database and these patients were used to provide a comparator group for any other patients that may test positive for U1RNP on further testing in Cluster 4.

Statistical Methods:

Principal component analysis ((PCA); Chapter 2, pp.72-75) was undertaken to determine if the variance within Cluster 4 may be explained by identifiable, underlying factors. Data were reviewed and presented in table form and graphically to illustrate differing AA profiles. We assessed whether there were unique clinical features among Cluster 4 patients according to their AA profile, both when compared within the Cluster 4 cohort and the entire SSc cohort (n=505).

Where numbers permitted, statistical analysis was used to explore any clinical associations with the SSc associated AAs. Statistical analyses were also undertaken to explore the clinical features of any patients testing positive to one of the three primary AA. To limit bias, only patients monospecific for the less specific or rarer disease associated AA were tested. Fisher's Exact method was used for discreet nominal variables. Continuous variables were assessed with ANOVA, using Levene's test for homogeneity of variances. To protect against a Type 1 error, if homogeneity of variances were violated, a Mann-Whitney U test was applied and this is annotated where undertaken. Results $p \leq 0.05$ are considered statistically significant. In some instances trends are reported where they have been found to be significant in other cohorts and are reported where $p<0.09$.

Results

Autoantibody Analysis

Frequency and Combination

The frequency of individual AAs and their expression either monospecifically or in combination is summarised in Table 4-1 and Figure 4-1. Forty-nine sera (34.5%) were monospecific, 29 (20.4%) had 2 AAs, five (3.5%) had 3 AAs, two (1.4%) had 4 AAs and one sera (0.7%) had 5 AAs. Fifty-six (39.4%) patients were negative for all SSc immunoblot AAs (Figure 4-2). Further exploration (reported below) revealed that these sera negative patients are partly explained by the presence of U1RNP, partly by ANA (only) positive patients and partly by true AA negative patients (see Chapter 5, pp.121-131).

Table 4-1: Autoantibody frequencies and combinations in cluster 4 patients, n=142

Autoantibody	TRIM21/Ro52	Ku	PmScl	Th/To	NOR90	Fib	RNAP3	CENP	Topo1
TRIM21/Ro52	14	2	12	6	2	2	3	8	9
Ku		3	0	0	0	1	0	0	0
PmScl			11	3	0	1	2	4	2
Th/To				3	0	0	0	1	0
NOR90					1	0	0	0	0
Fib						1	0	0	1
RNAP3							2	1	1
CENP								4	5
Topo1									5
Monospecific1	14	3	11	3	1	1	2	4	5
Multiple AA2	51	5	27	11	3	3	6	13	15

1 Monospecific expression of AAs without any other AA co-expression 2 Total numbers of patients with expression of each AA. Due to multiple co-expression (2 or more AA), these numbers are less than the sum of the AA

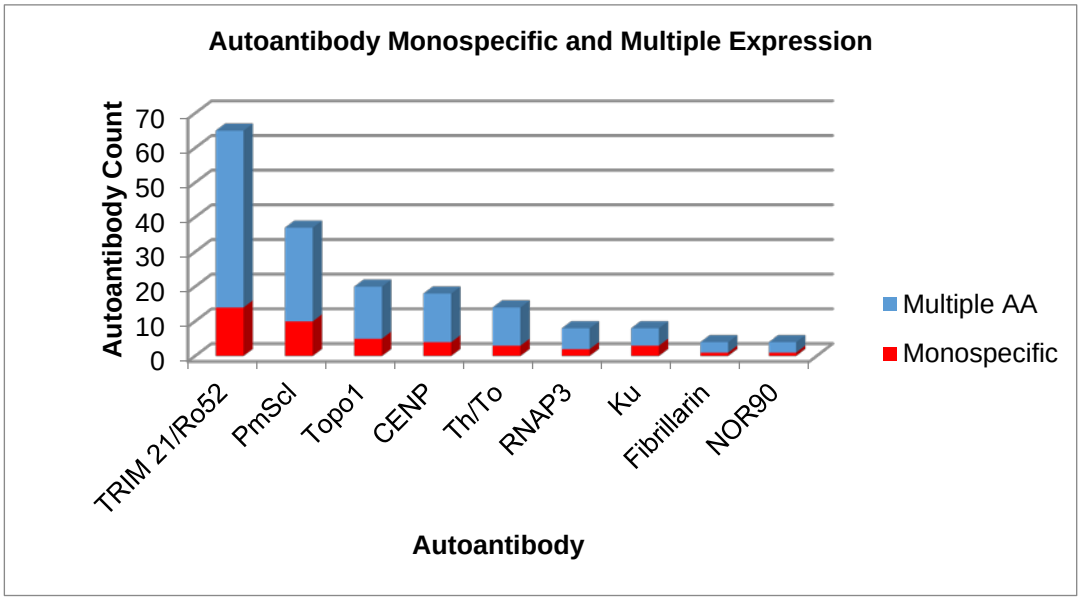


Figure 4-1: Individual autoantibody counts in Cluster 4 ASCS patients positive for at least one AA (n=86). Patients seronegative on Euroimmun assay are not included in this table. NOR90 - 90-kDa nucleolus organizer region, PM-Scl- polymyositis/scleroderma (exosome), TRIM21/Ro52 -tripartite motif containing 21, RNAP3 - RNA Polymerase III, Topo1 – Topoisomerase1, CENP – Centromere protein A and B. NOTE: Platelet derived growth factor receptor was not detected in this cluster

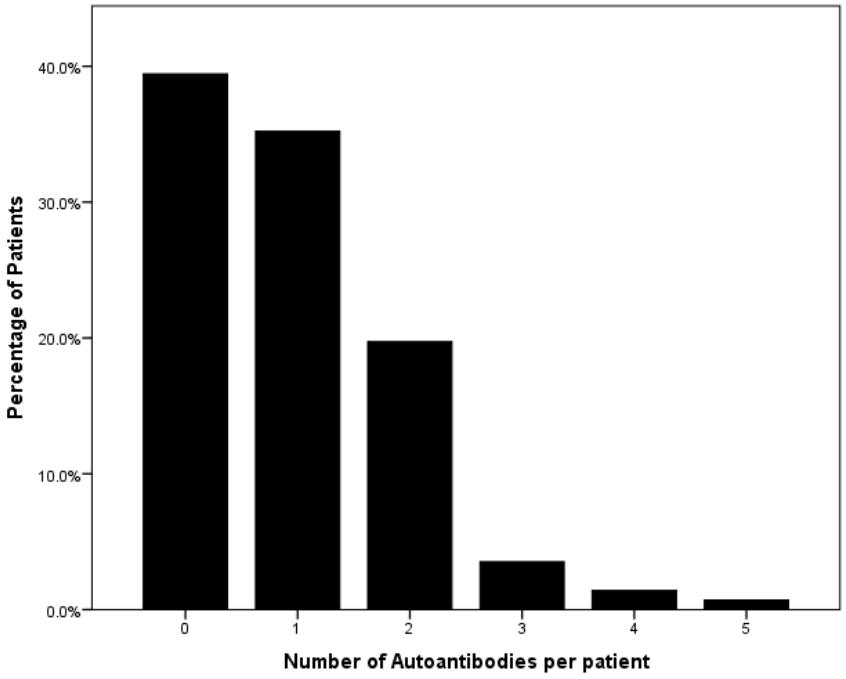


Figure 4-2: Cluster 4, Autoantibody expression per patient (%) utilising Euroimmun immunoblot.

Supplementary PCA

A detailed description of the supplementary PCA can be found in Chapter 2, Methodology (pp.72-75), however to summarise briefly; a scree plot in conjunction with Horn's parallel analysis determined that it was optimal to extract three components with any additional extractions not contributing meaningfully to the analysis. The strength of loadings can range from -1 to +1 signifying both positive and negative relationships between the AAs. Loadings ± .320 are considered to contribute to the relationship between variables (293). Following the PCA, a hierarchical cluster analysis revealed that unlike the initial PCA where the first two components demonstrated that 51.4% of the total variance was attributable to underlying factors, the relationships in Cluster 4 were much weaker, taking three extractions to show that 46.6% of the total variance was attributable to underlying factors. Nonetheless, using the data generated from the PCA in a hierarchical cluster analysis it was revealed that CENPB, Topo1, PmScl, Th/To and TRIM21/Ro52 accounted for the majority of relationships in Cluster 4. The remaining AAs; RNAP3, Ku, Fibrillarin and NOR90, were not present in sufficient numbers to determine any statistical significant outcome and to avoid a Type 1 error, further statistical analyses on these AAs were not done.

Expression of Topo1, CENP and RNAP3 in Cluster 4

The primary SSc AAs, CENP, Topo1 and RNAP3 were detected in this Cluster, either singularly or in various combinations with differing staining intensities. As these AAs had generally been captured by other clusters (see Chapter 3, pp.77-84) we reviewed their presence in this Cluster in detail to understand why they had not been captured in Clusters 1, 2, 3 or 5. Upon reviewing AA profiling (Table 4-2), it was evident that a combination of lower staining intensity, single epitope expression and AA co-expression has determined a place in Cluster 4 for these patients.

Topo1

- Topo1 appears 15 times
- Monospecific '+' 5 times (pts 23, 407, 759, 773, 948)
- Topo1 '+++' co-expressed with TRIM21/Ro52 '+++' and CENP B '++' twice (pt 115, 1050)
- Topo1 '++' co-expressed with TRIM21/Ro52 '+++' once (pt 126)
- Topo1 '+' co-expressed with CENP A '+++', TRIM21/Ro52 '+++' once (pt 328)
- Topo1 '+' co-expressed with CENP B '+' once (pt 266)
- Topo1 '+' co-expressed with TRIM21/Ro52 '+' once (pt 529)
- Topo1 '+' co-expressed with TRIM21/Ro52 '++' once (pt 252)
- Topo1 '+' co-expressed with TRIM21/Ro52 '+++' once (pt 1052)
- Topo1 '+' co-expressed with CENP B '+', RNAP3 155 '+', TRIM21/Ro52 '++',

PmScl ‘++’ once (pt 247)

- Topo1 ‘+’ co-expressed with TRIM21/Ro52 ‘+’. PM75 ‘+’, Fib ‘+’ once(pt 1221)

CENP A and B

- Appear 14 times
- CENP A ‘+++’ monospecific twice (pts 99, 851)
- CENP B ‘+’ monospecific twice (pts 194, 855)
- CENP A ‘+++’, co-expressed with TRIM21/Ro52 ‘+++’ and Topo1 ‘+’ once (pt 328)
- CENP A ‘+’ and CENP B ‘++’ co-expressed with PM75 ‘+’ once (pt 728)
- CENP A ‘++’, TRIM21/Ro52 ‘+++’ (pt 858)
- CENP A ‘+’, TRIM21/Ro52 ‘+++’ (pt 123)
- CENP B ‘++’ co-expressed with Topo1 ‘+++”, TRIM21/Ro52 ‘+++’ twice (pts 115, 1050)
- CENP B ‘++’, TRIM21/Ro52 ‘+++’, PM75 ‘++’ (pt 244)
- CENP B ‘+’ co-expressed with Topo1 ‘+’ once (pt 266)
- CENP B ‘+’ co-expressed with Topo1 ‘+’ and TRIM21/Ro52 ‘+++’, RNAP155 ‘+’ PM75 ‘++’ once (pt 247)
- CENP B ‘+’ co-expressed with TRIM21/Ro52 ‘++’, PM75 ‘++’, Th/To ‘+++’ once (pt 566)

RNAP3

- Appears 6 times
- RP11 ‘+’ monospecific once
- RP155 ‘+’ monospecific once
- RP11 ‘+’ co-expressed with TRIM21/Ro52 ‘+++’ twice
- RP11 ‘+’ co-expressed with PM75 ‘+’ once
- RP155 ‘+’ co-expressed with TRIM21/Ro52 ‘++’ and PM75 ‘++’ once

Table 4-2: Summary; Primary SSc autoantibodies and co-expressed AAs in Cluster 4,'Other', and their associated staining intensity.

Patient	Topo 1			CENP A			CENP B		RNAP11	RNAP155	TRIM21/Ro52			PM75		Th/To	Fib
	+	++	+++	+	++	+++	+	++	+	+	+	++	+++	+	++	+++	+
23	✓																
99						✓											
115			✓					✓					✓				
123				✓									✓				
126		✓											✓				
194							✓										
218									✓				✓				
244								✓					✓		✓		
247	✓						✓			✓		✓			✓		
252	✓											✓					
266	✓						✓										
328	✓					✓							✓				
380										✓							
407	✓																
529	✓										✓						
566							✓					✓			✓	✓	
725									✓				✓				
728				✓				✓						✓			
759	✓																
773	✓																
778									✓								
851						✓											
855							✓										
858					✓								✓				
914									✓					✓			
948	✓																
1050			✓					✓					✓				
1052	✓												✓				
1221	✓										✓			✓			✓

Comparisons; Topo1, CENP, RNAP3 and Topo1/CENP positive patients

The small number of patients positive for these AAs in Cluster 4 means that all results are reported with caution. As these three primary AA are disease specific and have been shown to be associated with specific clinical features in the majority of our tested patients, we were interested to assess if the presence of any of these three AA in Cluster 4, even when their expression was non-dominant, differed in their clinical characteristics to those expressing these AAs but captured in one of the other clusters. Patients positive for each of the primary SSc AAs in Cluster 4 'Other', regardless of any other AA expression, were compared to all patients in the cohort (see Chapter 3, pp. 77-84) positive for that specific AA. In addition, all double positive CENP/Topo1 patients (regardless of cluster association) were compared to either CENP positive or Topo1 positive patients

Topo1 Cluster 4 (n=15) vs all Topo1 (n=97)

15/112 patients positive for Topo1 were in Cluster 4 'Other', 4/112 were in Cluster 5, CENP, and the remainder were in Cluster 3, Topo1. Comparative analyses demonstrated four significant differences when comparing Topo1 positive patients in Cluster 4 with all other Topo1 patients.

i. Patients with Topo1 in Cluster 4 were more likely to be male (46.7% (7/15) vs 19.6% (19/97); Fishers Exact p=0.043).
ii. PAH was more common among Cluster 4 Topo1 patients (4/15 or 26.7%) compared with other Topo1 patients (5/97 or 5.2%; Fishers Exact p= 0.018).
iii. Fewer Topo1 patients in Cluster 4 (6/15 or 40% vs 69/97 or 71.1%) had ILD (Fishers Exact p= 0.035).
iv. Oesophageal dysmotility was more common in Cluster 4 Topo1 patients (4/15 or 26.7%) compared with Topo1 patients in other Clusters (7/97 or 7.2%; Fishers Exact p=0.04).

All other demographic, clinical and serological variables were statistically comparable.

CENP Cluster 4 (n=14) vs all CENP (n=214)

For this analysis, the presence of CENP A and/or CENP B was considered CENP positive. There were 228/505 patients positive for CENPA/B; 206/228 were in Cluster 5 CENP; 6/228 were in Cluster 3, Topo1; 1/228 in Cluster 1, RNAP3 'strong'; 1/228 in Cluster 2, RNAP3 'weak' and the remaining 14/228 patients were in Cluster 4, 'Other'. There were three significant differences observed when comparing Cluster 4 CENP patient with other CENP patients.

i. There were more males with CENP in Cluster 4 (4/14, 28.6%) compared to other CENP positive patients (13/214, 6.1%) (Fishers Exact p=0.013).
ii. Oesophageal dysmotility was experienced by more CENP positive patients (Fishers Exact p=0.028) in Cluster 4 (5/14 or 35.7%) compared with other CENP positive patients (26/214 or 12.1%).

Cluster 4 RNAP3 (n=6) vs all RNAP3 (n=75)

For this analysis, RNAP3 was considered to be present if one or both epitopes were detected. 81/505 patients were positive for RNAP3, 39/81 in Cluster 1 RNAP3 'strong'; 24/81 in Cluster 2 RNAP3 'weak'; 10/81 in Cluster 5 CENP, 2/81 in Cluster 3 Topo1, and 6/81 in Cluster 4 'Other'. There were no significant differences between RNAP3 positive patients in Cluster 4 and other RNAP3 positive patients.

These results are summarised in Table 4-3.

Table 4-3: Summary comparison of data Cluster 4 primary autoantibody data

	Cluster 4 Topo1 patients compared with Topo1 Patients			Cluster 4 CENP patients compared with CENP Patients			Cluster 4 RNAP3 Patients compared with all RNAP3 patients		
	Cluster 4 Topo1 Positive n=15	All Topo1 Positive n=97	p	Cluster 4 CENP Positive n=14	All CENP Positive n=214	p	Cluster 4 RNAP3 n=6	All RNAP3 patients n=75	p
	% (n) or mean ± SD	% (n) or mean ± SD	Fishers Exact or # Mann Whitney U	% (n) or mean ± SD	% (n) or mean ± SD	Fishers Exact or # Mann Whitney U	% (n) or mean ± SD	% (n) or mean ± SD	Fishers Exact or #Mann Whitney U
Demographic variables									
Males	46.7% (7)	19.6% (19)	**0.043**	28.6% (4)	6.1% (13)	**0.013**	0%	9.3% (7)	NS‡
Age first Raynaud's (years)	40.53 ± 13.88	40.48 ±13.37	NS	38.34 ± 11.78	39.26 ± 15.84	NS	42.08 ± 10.8	46.5 ± 15.35	NS
Age onset disease (years)	42.95 ± 13.25	42.72 ± 13.95	NS	41.46 ± 10.06	46.56 ± 13.82	NS # 0.089	42.33 ± 10.93	46.97 ± 10.5	NS
Disease subset									
Limited cutaneous disease	60% (9)	51.5% (50)	NS	85.7% (12)	93.9% (201)	NS	50% (3)	34.7% (26)	NS
Diffuse cutaneous disease	40% (6)	48.5% (47)	NS	14.3% (2)	6.1% (13)	NS	50% (3)	65.3% (49)	NS
Clinical Variables									
Pulmonary Arterial Hypertension	26.7% (4)	5.2% (5)	**0.018**	14.3% (2)	16.4% (35)	NS	-	-	-
Interstitial Lung Disease	40% (6)	71.1% (69)	**0.035**	35.7% (5)	15.9% (34)	NS 0.069	-	-	-
Oesophageal dysmotility	26.7% (4)	7.2% (7)	**0.04**	35.7% (5)	12.1% (26)	**0.028**	-	-	-
Modified Rodnan Skin Score	15.2 ± 11.76	16.44 ± 9.6	NS	9.91 ± 9.03	9.95 ± 6.97	NS	14.33 ± 11.22	21.51 ± 12.51	NS

‡ Not Significant

Co-Expression of Topo1 and CENP:

Fifteen patients were identified as co-expressing (double positive) Topo1 and CENP in the Clusters identified in Chapter 3 (n=505). Five were in Cluster 4 'Other', six were in Cluster 3 Topo1 and four were in Cluster 5 CENP. Cluster allocation by PCA and staining intensity for both Topo1/CENP and any co-expressed AAs can be seen in Table 4-4.

Previously these AA were felt to be mutually exclusive, and we wished to explore if there were any unique clinical associations when patients were dual positive. Therefore, we have compared these double positive patients from the cohort as a whole, rather than limiting analysis to those who were only identified in Cluster 4. We compared these double positive patients to both Topo1 and CENP patients to explore similarities and differences between the groups.

Table 4-4: CENP/Topo1 double positive patients, cluster allocation, staining intensity and other co-expressed AAs

		Cluster	TRIM21/Ro52			PM75			PM100	NOR90	RNAP155	CENP A		CENP B			Topo 1		
			1	2	3	1	2	3	1	1	1	1	3	1	2	3	1	2	3
Patient	47	3, Topo1						✓				✓							✓
	107	5, CENP						✓	✓				✓			✓	✓		
	115	4, Other			✓										✓				✓
	124	3, Topo1					✓							✓					✓
	247	4, Other		✓			✓				✓			✓			✓		
	266	4, Other												✓			✓		
	328	4, Other			✓								✓				✓		
	426	3, Topo1					✓								✓				✓
	442	5, CENP						✓					✓			✓	✓		
	543	3, Topo1			✓		✓							✓					✓
	661	5, CENP			✓					✓			✓			✓		✓	
	905	3, Topo1					✓							✓					✓
	960	3 Topo1	✓			✓								✓					✓
	1050	4, Other			✓										✓				✓
	1119	5, CENP											✓			✓	✓		

Co-expression of Topo1/CENP (n=15) compared with Topo1 (n=97)

There were two significant differences when comparing double positive patients to Topo1 patients:

i) All double positive patients (100%) experienced a dry mouth (Fishers Exact p=0.011) compared to 68/97 (70.1%)

ii) Double positive patients were younger for age onset Raynaud's (Mann Whitney U test p=0.045). The median and interquartile range can be seen in Figure 4-3 and Table 4-5.

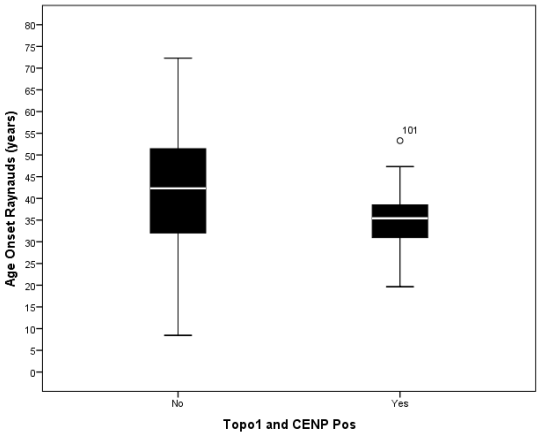


Figure 4-3: Age onset Raynaud's Comparison; Topo1/CENP double positive vs Topo1 patients.
NOTE: Medians are used in Mann Whitney Box & Whisker Plots. The median is noted as the horizontal line in each box and whisker plot. For each variable measured, the interquartile range (IQR) is the spread of data from the median.

Table 4-5: IQR for double positive Topo1/CENP vs Topo1 patients for Age of Onset Raynaud's

	AA status	Median	Interquartile Range
Age Onset Raynaud's (years)	Double positive	35.40	30.01 - 40.79
	Topo1	41.93	32.04 - 51.82

Co-expression of Topo1/CENP (n=15) compared with CENP (n=213)

There were seven significant differences seen between double positive patients compared with CENP patients. Double positive patients are more likely to be male (40% vs 5.2%; Fishers Exact p=0.000); have more diffuse disease (26.7% vs 5.2%; Fishers Exact p=0.011), and a younger age of disease onset (Mann Whitney U test p=0.015) (Table 4-6, Figure 4-4). Furthermore, Topo1/CENP positive patients were more likely to have ILD (46.7% vs 15%; Fishers Exact p=0.006); less likely to have systemic hypertension (20% vs 52.6%; Fisher Exact p=0.017); less likely to have anal incontinence (6.7% vs 44.6%; Fishers Exact p=0.005) and their highest recorded serum CK was greater (165.87± 113.47 vs 114.27± 90.08; Mann Whitney U test p=0.034) as compared with CENP patients.

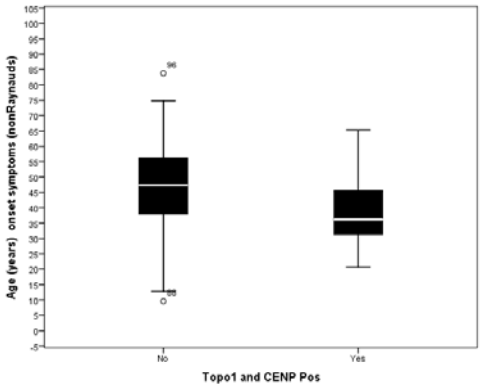


Figure 4-4: Age comparison disease onset Topo1/CENP double positive vs CENP patients

NOTE: Medians are used in Mann Whitney Box & Whisker Plots. The median is noted as the horizontal line in each box and whisker plot. For each variable measured, the interquartile range (IQR) is the spread of data from the median.

Table 4-6: IQR for double positive Topo1/CENP vs CENP patients; Age Disease Onset

	AA status	Median	Interquartile Range
Age Disease Onset (years)	Double positive	36.18	27.50 - 44.86
	CENP	47.37	38.16 - 56.58

A summary of all significant results for double positive Topo1/CENP patient comparisons can be found in Table 4-7.

Table 4-7: Summary comparison co-expression of Topo1/CENP data

	All Co-expressed Topo1/CENP Patients compared with All Topo1 Patients			All Co-expressed Topo1/CENP Patients compared with CENP Patients		
	Co-expressed Topo1/CENP Positive n=15	All Topo1 Positive n=97	p	Co-expressed Topo1/CENP Positive n=15	All CENP Positive n= 213	p
	% (n) or mean ± SD	% (n) or mean ± SD	Fishers Exact or #Mann Whitney U	% (n) or mean ± SD	% (n) or mean ± SD	Fishers Exact or # Mann Whitney U
Demographic variables						
Males	40% (6)	20.6% (20)	NS‡	40% (6)	5.2% (11)	**0.00**
Age first Raynaud's (years)	35.03 ± 9.55	41.31 ± 13.71	**# 0.045**	35.03 ± 9.55	39.95 ± 16.11	NS
Age onset disease (years)	36.96 ± 12.47	43.63 ± 13.83	NS	38.51 ± 13.43	47.26 ± 13.84	**# 0.015**
Disease subset						
Limited cutaneous disease	73.3% (11)	49.5% (48)	NS	73.3% (11)	94.8% (202)	NS
Diffuse cutaneous disease	26.7% (4)	50.5% (49)	NS	26.7% (4)	5.2% (11)	**0.011**
Clinical Variables and Serology						
Interstitial Lung Disease	46.7% (7)	70.1% (68)	NS 0.084	46.7% (7)	15% (32)	**0.006**
Systemic Hypertension	-	-	-	20% (3)	52.6% (112)	**0.017**
Dry Mouth	100% (15)	70.1% (68)	**0.011**	-	-	-
Anal incontinence	-	-	-	6.7% (1)	44.6% (95)	**0.005**
Serum CK	-	-	-	165.87 ± 113.47	114.27± 90.08	**# 0.034**
Modified Rodnan Skin Score	14.35 ± 9.72	16.55 ± 9.92	NS	13.8± 9.62	9.75 ± 6.94	- NS

‡ Not significant

TRIM21/Ro52

Fourteen Cluster 4 patients were monospecific for TRIM21/Ro52 and this was the most frequently expressed AA either monospecifically or in multiple combinations in this Cluster. Cluster 4 patients monospecific for TRIM21/Ro52 were compared to the ASCS cohort excluding patients that co-expressed this AA.

Following this analysis, monospecific TRIM21/Ro52 was then compared to other patients in Cluster 4, again excluding patients with TRIM21/Ro52 co-expression.

Whole cohort comparison, excluding TRIM21/Ro52 positive patients (n=334) compared with Cluster 4 TRIM21/Ro52 (n=14)

There were three significant differences between monospecific TRIM21/Ro52 patients in Cluster 4 and those negative for TRIM21/Ro52 in the remainder of the cohort. Monospecific patients experienced first Raynaud's later (p= 0.044) than their negative counterparts. They also experienced a higher erythrocyte sedimentation rate (ESR) (Mann Whitney U p= 0.024) and a higher systolic pulmonary arterial pressure (sPAP) (mmHG) (43.92+/-10.77 vs 37.48+/-14.32; Mann Whitney U p=0.022)), In this analysis of monospecific TRIM21/Ro52 patients, there was no increase in PAH compared with the remainder of the cohort

These results and a comparison of monospecific TRIM21/Ro52 positive and negative patients within Cluster 4 are summarised in Table 4.7.

Cluster 4 comparison (n=105) with Cluster 4 TRIM21/Ro52 patients (n=14)

TRIM21/Ro52 monospecific patients first experienced Raynaud's at an older age (p=0.042) than the remainder patients in Cluster 4.

Table 4-8: Comparison TRIM21/Ro52 monospecific and TRIM21/Ro52 negative patients

	Whole cohort			Cluster 4		
Demographic variables	TRIM21/Ro52 monospecific (n=14) % (n) or mean ± SD	TRIM21/Ro52 Negative (n=320) % (n) or mean ± SD	p value Fishers Exact, ANOVA # Mann-Whitney U	TRIM21/Ro52 monospecific (n=14) % (n) or mean ± SD	TRIM21/Ro52 Negative (n=91) % (n) or mean ± SD	p value Fishers Exact ANOVA # Mann-Whitney U
Females	71.4% (10)	86.6% (277)	NS ‡	71.4 (10)	81.3 (74)	NS
Age first Raynaud's (years)	49.07 ± 14.93	40.64 ± 15.29	**0.044**	49.07 ± 14.93	37.02 ± 21.04	**0.042**
Age onset disease (years)	50.21 ± 14.83	45.02 ± 13.73	NS	50.21 ± 14.83	43.50 ± 15.93	NS
Disease subset						
Limited cutaneous disease	64.3% (9)	69.1% (221)	NS	64.3% (9)	76.9% (70)	NS
Diffuse cutaneous disease	35.7% (5)	30.9% (99)	NS	35.7% (5)	23.1% (21)	NS
Clinical Variables and Serology						
Erythrocyte sedimentation rate	40.43 ± 26.11	25.73 ± 18.50	**0.024 #**	40.43 ± 26.11	27.66 ± 18.42	NS 0.070 #
Systolic pulmonary arterial pressure (mm Hg)	43.92 ± 10.77	37.48 ± 14.32	**0.022 #**	43.92 ± 10.77	38.29 ± 13.73	NS 0.052 #
Abnormal ECG result	80% (8)	46.9% (107)	NS 0.053	80% (8)	47.5% (28)	NS 0.087
Myocardial disease	7.1% (1)	0.3% (1)	NS 0.082	7.1% (1)	0	NS
Interstitial Lung Disease	57.1% (8)	34.7% (111)	NS 0.095	57.1% (8)	29.7% 27	NS 0.065
Digital Ulcers - No	71.4% (10)	46.3% (148)	NS 0.098	71.4% (10)	51.5% (47)	NS
Modified Rodnan Skin Score	13.57 ± 7.55	13.70 ± 10.18	NS	13.57 ± 7.55	10.96 ± 9.16	NS

‡ Not significant

PmScl

Eleven female patients were monospecific for PmScl. Seven of these patients were monospecific for PM75, two for PM100 and two patients had both epitopes. It was most often co-expressed with TRIM21/Ro52 but it was not uncommon to be co-expressed with other AAs, particularly CENP and to a lesser extent Topo1, RNAP3 and Th/To. Due to the low number of patients with one or both epitopes, monospecific PmScl was defined as being positive for either or both epitopes with no co-expression of other AAs.

Comparisons were made between PmScl monospecific patients and PmScl negative patients in the entire ASCS cohort and also within Cluster 4, (Table 4-9).

Whole cohort comparison (n= 427) with Cluster 4 PmScl (n=11)

There were two statistically significant differences found in this cohort. PmScl monospecific patients experienced their first Raynaud's episode (p= 0.004) and disease onset (p=0.029) at a younger age compared to PmScl negative patients (Table 4-9). There were no other detectable clinical differences between the groups and in particular, no difference in the presence of myositis, calcinosis, ILD or gastrointestinal disturbances.

Cluster 4 comparison (n= 127) with Cluster 4 PmScl (n=11)

There were no significant differences between PmScl monospecific patients and PmScl negative patients in Cluster 4 (Table 4-9).

Table 4-9: Comparison; PmScl monospecific and negative patients

	Whole cohort			**Cluster 4**		
Demographic variables	PmScl Monospecific n=11 % (n) or mean ± SD	PmScl negative n=494 % (n) or mean ± SD	p value Fishers Exact or ANOVA # Mann-Whitney U	PmScl Monospecific n=11 % (n) or mean ± SD	PmScl negative n= 116 % (n) or mean ± SD	p value Fishers Exact or ANOVA # Mann Whitney U
Females	100% (11)	86.5% (360)	NS ‡	100% (11)	76.4% (89)	NS
Age first Raynaud's (years)	28.35 ± 16.61	42.07 ± 15.65	**0.004**	28.35 ± 16.61	38.83 ± 20.83	NS 0.058 #
Age onset disease (years)	36.92 ± 14.35	46.42 ± 14.15	**0.029**	36.92 ± 14.35	45.08 ± 16.09	NS 0.083 #
Disease subset						
Limited cutaneous disease	81.8% (9)	71.4 (297)	NS	81.8% (9)	75% (87)	NS
Diffuse cutaneous disease	18.2% (2)	28.6% (119)	NS	18.2% (2)	25% (29)	NS
Clinical Variables						
Modified Rodnan Skin Score	12.40 ± 10.04	13.37 ± 10.12	NS	12.40 ± 10.04	11.48 ± 9.48	NS

‡ NS Not Significant

U1RNP

U1RNP has been included as a supplementary analysis as it is a significant AA found in SSc patients. It is not included on the Euroimmun SSc Immunoblot, but it is available on the Euroimmun ANA profile 5 Immunoblot. Patients positive for U1RNP are frequently seen as 'overlap' patients and have features of both SSc and other connective tissue diseases and are therefore an important part of the SSc spectrum (22, 234).

Some patients in the ASCS had previously tested positive for U1RNP and are classified as SSc. There were a large number of patients with a negative result in the initial SSc blot. Therefore, we decided to test selected patients to determine if some of those SSc 'blot negative' patients were indeed negative or were positive for this known and SSc associated AA. A total of 80 selected patients were retested for U1RNP in Cluster 4 using the ANA profile 5 line blot based on their sera having one or more of the following criteria;

i. Speckled ANA.
ii. Negative results in the SSc line blot but with a previous U1RNP positive test result.
iii. Positive monospecific for TRIM21/Ro52 SSc blot result.
iv. Sufficient sera were available for testing.

For a detailed explanation of the methods, please refer to Chapter 2, Methodology pp 68.

To keep this analysis consistent with the above analyses, U1RNP positive patients will be compared to all other patients in the ASCS cohort and also with patients within Cluster 4. However, this analysis will differ so that in line with the literature where U1RNP confers an 'overlap' variant, patients are analysed on an AA positive/negative status.

Results U1RNP Testing Individual U1RNP patient data

Among the 80 patients that were U1RNP blot tested in Cluster 4, 56 were blot negative on initial Scleroderma Euroimmun blot testing. One SSc blot negative patient was not retested by the U1RNP blot as no serum was available and no further patient data were available. Therefore, for the purpose of this analysis they were considered U1RNP negative. Fourteen patients were TRIM21/Ro52 monospecific and 29 had a speckled ANA. Table 4-10 summarises the results of all U1RNP blot testing criteria (as explained above) in Cluster 4.

Table 4-10: Summary, Cluster 4 U1RNP blot testing criteria and results

		SSc blot antibody	Blot U1RNP Tested	ANA positive ASIG	ANA speckled ASIG	Blot U1RNP	RNP ASIG
		✓ Negative/TRIM21 mono	Yes	Yes	Yes	Positive	Yes
Patient	13	✓	✓	✓	✓		
	18	✓	✓				
	35	TRIM21/Ro52 mono	✓				
	36	✓	✓	✓	✓		
	37	✓	✓	✓	✓		
	53	✓	✓				
	58	✓	✓	✓			
	60	✓	✓	✓			
	64	✓	✓	✓			
	66	✓	✓				
	77	TRIM21/Ro52 mono	✓	✓	✓		
	82	✓	✓	✓			
	83	✓	✓	✓			
	114	✓	✓	✓	✓		
	139	TRIM21/Ro52 mono	✓	✓			
	140	✓	No serum available				
	163	✓	✓				
	194		✓				
	199	TRIM21/Ro52 mono	✓				
	212	✓	✓	✓	✓		
	274	✓	✓	✓		✓	
	278	✓	✓	✓			
	288	TRIM21/Ro52 mono	✓	✓	✓	✓	
	314	✓	✓	✓			
	323	TRIM21/Ro52 mono	✓				
	325	TRIM21/Ro52 mono	✓	✓	✓	✓	
	326	TRIM21/Ro52 mono	✓				
	327	✓	✓				
	333	TRIM21/Ro52 mono	✓	✓	✓	✓	
	345	✓	✓				
	346	✓	✓	✓			
	347	✓	✓				
	354	✓	✓	✓			
	355	✓	✓	✓	✓		
	358	✓	✓	✓			
	374	✓	✓	✓	✓		
	381		✓	✓			
	398	✓	✓	✓	✓	✓	✓
	407		✓	✓			
	441	✓	✓	✓			
	451	✓	✓	✓	✓		
	459	TRIM21/Ro52 mono	✓	✓			
	472	✓	✓				
	481	✓	✓				
	507	TRIM21/Ro52 mono	✓	✓	✓		
	522	✓	✓				
	529		✓	✓			
	557	✓	✓	✓			
	560	✓	✓	✓		✓	
	617	TRIM21/Ro52 mono	✓	✓			
	632	✓	✓	✓	✓		
	637	✓	✓	✓	✓		
	638	✓	✓				
	643	✓	✓	✓			
	654	✓	✓	✓	✓	✓	✓
	688	✓	✓	✓			
	700	✓	✓	✓	✓	✓	✓
	742	TRIM21/Ro52 mono	✓	✓	✓	✓	✓
	743	✓	✓	✓	✓		
	775	✓	✓				
	802	✓	✓				
	807	✓	✓				
	810	✓	✓	✓	✓	✓	✓

	Any Blot Antibody	Blot U1RNP Tested	ANA Positive ASIG	ANA Speckled ASIG	Blot U1RP	RNP ASIG
	✓ Negative/TRIM21 mono	Yes	Yes	Yes	Positive	Yes
837	✓	✓	✓	✓		
852	TRIM21/Ro52 mono	✓	✓	✓	✓	✓
854	✓	✓				
855		✓	✓			
888	✓	✓	✓			
892	TRIM21/Ro52 mono	✓				
894	✓	✓	✓			
924	✓	✓	✓			
953	✓	✓				
974	TRIM21/Ro52 mono	✓	✓	✓		
990	TRIM21/Ro52 mono	✓	✓	✓		
1022	✓	✓				
1046	TRIM21/Ro52 mono	✓	✓	✓		
1052		✓	✓	✓		
1059	✓	✓	✓	✓		
1100	TRIM21/Ro52 mono	✓				
1110	✓	✓				
1124	✓	✓	✓	✓		

Eleven sera from Cluster 4 were identified positive for U1RNP by ANA profile 5 LIA. 6/11 patients with an initial negative SSc blot result had previously tested positive for U1RNP (by ASIG independent laboratories), and 5/11 patients had a monospecific TRIM21/Ro52 blot result. Upon retesting, all patients were U1RNP LIA blot positive.

Revised blot testing outcomes and the original ASIG results can be seen in Table 4-10. At the time of the original blot testing all patients in this subgroup fulfilled the 1980 ACR SSc criteria or the Medsger criteria. Recently the patients were reassessed using the 2013 ACR/EULAR revised SSc classification criteria and all were classified as having SSc (Table 4-11).

The first non-Raynaud's symptom for 7/11 patients was skin involvement, for 2/11 patients it was inflammatory arthritis and for the final 2/11 it was pleuro-pericarditis (Table 4-12). All clinical manifestations for each patient can be seen in Table 4-13, of note, all patients experienced Raynaud's phenomenon, 10/11 had dilated nailfold capillaries, 9/11 had telangiectasias and 8/11 had been, or were smokers.

Table 4-11: Results of retesting selected patients for U1RNP Autoantibodies compared with the results of the initial ASIG autoantibody testing

		BLOT TRIM21/Ro52		***BLOT*** U1RNP A			***BLOT*** U1RNP C			***BLOT*** U1RNP 70		***BLOT*** Sm		***BLOT*** U1RNP_Sm			***ASIG*** RNP	***ASIG*** Ro	***ASIG*** La	***ASIG*** Sm	***ASIG*** ANCA	***ASIG*** RF	***ASIG*** APL	***ASIG*** LAC
		++	+++	+	++	+++	+	++	+++	++	+++	+	++	+	++	+++	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Patient	274				✓											✓								
	288		✓											✓										
	325	✓		✓						✓						✓								
	333		✓											✓				✓	✓			✓	✓	✓
	398						✓										✓							
	560														✓									
	654					✓					✓					✓	✓							
	700			✓								✓				✓	✓				✓	✓	✓	
	742		✓			✓										✓	✓	✓		✓				
	810				✓				✓		✓		✓			✓	✓							
	852		✓			✓		✓		✓						✓	✓	✓						

Table 4-12: Classificaton criteria and relevant clinical details for 11 U1RNP SSc patients

Patient	1980 ACR criteria filled		Medsger Criteria Filled	1st clinical non Raynaud's symptom			Highest Modified Rodnan Skin Score	SSc Classification	Disease Duration Years	ANA Pattern
	No	Yes	Yes	Inflammatory Arthritis	Pleuro-pericarditis	Skin				
274		✓				✓	24	LSSc	8.1	Centromere
288		✓				✓	7	LSSc	5.2	Speckled
325		✓		✓			9	LSSc	7.6	Speckled
333		✓				✓	14	dcSSc	2.8	Speckled Nucleolar
398		✓				✓	9	LSSc	13.3	Speckled
560		✓				✓	24	dcSSc	21.7	Homogeneous
654		✓		✓			22	dcSSc	26.1	Speckled
700		✓			✓		4	LSSc	37.9	Speckled
742		✓				✓	4	LSSc	9.3	Speckled
810	✓		✓			✓	Missing	LSSc	6.9	Speckled
852		✓	✓		✓		7	LSSc	5.2	Speckled

Table 4-13: Clinical manifestations for all blot positive U1RNP SSc patients. (note only clinically positive data shown)

Patient	ILD	PAH	ECG Result	EGC RBBB	Echo Lv systolic Abnormal	C3 below normal	C4 below normal	Telangectasia	Calcinosis	Dry mouth	Dry eyes	Reflux oesophagitis	Synovitis definite	Digital ulcers	Digital gangrene	Smoked	Raynaud's	Systemic hypertension	Digital amputation	Nailfold Capillary	Tendon friction rubs	Joint contractures
	Ever	Ever	Abnormal	Ever	Abnormal	Ever	Ever	Ever	Ever	Ever	Ever	Definite	Ever	Ever	Ever	Ever	Ever	Ever	Ever	Ever	Ever	Ever
274					✓	✓			✓	✓	✓	✓		✓		✓	✓					
288	✓		✓					✓	✓	✓	✓	✓	✓			✓	✓	✓		✓		✓
325								✓	✓							✓	✓			✓		✓
333							✓	✓	✓			✓		✓			✓			✓		✓
398					✓	✓		✓	✓		✓	✓				✓	✓	✓		✓		✓
560								✓	✓	✓				✓	✓		✓		✓	✓	✓	✓
654								✓	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓		✓
700		✓	✓	✓	✓			✓		✓	✓			✓	✓	✓	✓	✓	✓	✓		
742						✓	✓	✓		✓	✓					✓	✓			✓		
810						✓	✓			✓	✓			✓			✓			✓		
852						✓		✓			✓				✓	✓	✓	✓		✓	✓	

Whole cohort comparison (n= 494) with U1RNP positive (n=11)

There were seven statistically significant differences found between U1RNP positive Cluster 4 patients and the remainder of the cohort. Patients who were U1RNP positive experienced Raynaud's (p=0.040) and first non-Raynaud's symptoms (p=0.014) at a younger age. Males represented 45.4% of the Cluster 4 U1RNP positive group but only 11.5% of negative patients (Fishers Exact p=0.006). Digital gangrene was present in 36.4% of positive patients compared with 9.9% of negative patients (Fishers Exact p=0.020), no patients with U1RNP experienced anal incontinence compared to 31.8% of U1RNP negative patients (Fishers Exact p=0.021) and left ventricular ejection fraction (LVEF) was higher (p=0.023) in U1RNP patients. Serologically, U1RNP positive patients had a history of reduced C3 in 45.5% of patients compared with 13% of negative patients (Fishers Exact p= 0.01).

Cluster 4 comparison (n= 131) with U1RNP positive (n=11)

The within Cluster comparison was similar to the whole cohort comparison with most of the statistically significant differences remaining. Patients again experienced the first non-Raynaud's symptoms earlier (p=0.049) with males representing 45.5% of the U1RNP positive patients compared with 18.3% of the negative patients (Fishers Exact p=0.047). C3 was below normal in 45.5% of the U1RNP positive patients compared to 14.5% of U1RNP negative patients (Fishers Exact p=0.021) and 36.4% of U1RNP patients experienced digital gangrene compared with 8.4% negative patients (Fishers Exact p=0.017).

Table 4-13 shows a comparison between U1RNP positive and negative patients for the entire cohort and also within Cluster 4.

Table 4-14: Comparison U1RNP positive and negative patients, whole cohort and Cluster 4

	Whole cohort			Cluster 4		
Demographic variables	U1RNP Positive n=11 % (n) or mean ± SD	U1RNP negative n=494 % (n) or mean ± SD	p value Fishers Exact or ANOVA	U1RNP Positive n=11 % (n) or mean ± SD	U1RNP negative n= 131 % (n) or mean ± SD	p value Fishers Exact or ANOVA
Males	45.5% (5)	11.5% (57)	**0.006**	45.5% (5)	18.3% (24)	**0.047**
Age first Raynaud's (years)	31.74 ± 14.22	41.62 ± 15.75	**0.040**	31.74 ± 14.22	39.02 ± 20.31	NS ‡
Age first non-Raynaud's symptom (years)	35.72 ± 18.38	46.24 ± 13.89	**0.014**	35.72 ± 18.38	45.36 ± 15.19	**0.049**
Disease subset						
Limited cutaneous disease				72.7% (8)	77.9% 102)	NS
Diffuse cutaneous disease				27.3% (3)	22.1% (29)	NS
Clinical Variables and Serology						
C3 Below Normal (ever)	45.5% (5)	13% (54)	**0.010**	45.5% (5)	14.5% (19)	**0.021**
Digital Gangrene	36.4% (4)	9.9% (40)	**0.020**	36.4% (4)	8.4% (11)	**0.017**
Anal Incontinence	0	31.8% (157)	**0.021**	0	24.4% (32)	NS 0.070
Lowest LVEF (%)	67.25 ± 9.32	60.42 ± 8.36	**0.023**	67.25 ± 9.32	60.70 ± 9.35	NS 0.058
Modified Rodnan Skin Score	12.40 ± 8.07	13.04 ± 9.95	NS	12.40 ± 8.07	10.94 ± 9.26	NS

‡ NS Not significant

U1RNP SSc patients compared with U1RNP Mixed Connective Tissue Disease (MCTD) patients.

The Australian Scleroderma Cohort Study database includes 21 patients classified as MCTD where sera were available and who had undergone Euroimmun testing. These patients' sera are used in this supplementary, univariate comparative analysis to compare with the 11 patients that are U1RNP positive and classified as SSc.

Both subgroups were statistically comparable for all clinical variables with the exception of two significant differences. MCTD patients had a lower modified Rodnan Skin Score (7.81± 6.67 vs 16.00 ± 12.94 Mann Whitney U ^{exact} p=0.047) when compared with SSc patients (Figure 4-8).

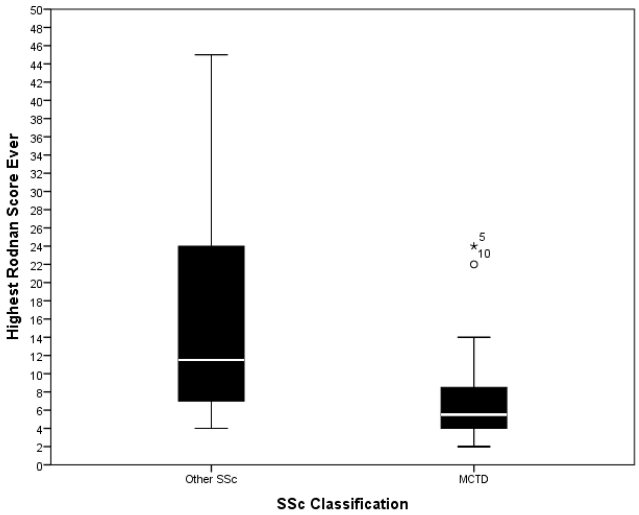


Figure 4-8: MRSS comparison U1RNP positive SSc
(Median 11.50, IQR 2.5 – 20.5) and MCTD (Median 5, IQR 2.5 – 7.5) patients.

NOTE: Medians are used in Mann Whitney Box & Whisker Plots. The median is noted as the horizontal line in each box and whisker plot. For each variable measured, the interquartile range (IQR) is the spread of data from the median.

No U1RNP positive MCTD patients had digital gangrene whereas 4/11 (36.4%) of U1RNP positive SSc patients experienced digital gangrene (Fishers Exact p=0.009). However, one of these patients also had anti-phospholipid syndrome which may have biased results.

Discussion

Our initial work demonstrated that in the majority of cases where a primary SSc associated AA is present, that AA can be used to guide sub classification of disease, independent of clinical findings. However, SSc patients who lack these primary disease specific AAs or who express them in a mixed or non-dominant fashion may present a significant diagnostic challenge to clinicians. Our initial analysis confirmed that these patients make up a substantial number of those seen in the ASCS registry. Therefore, we were interested to evaluate this challenging group further to determine if any AA could provide any further aid to clinical assessment in this setting. The PCA in a hierarchical cluster analysis revealed that CENP B, Topo1, PmScl, Th/To and TRIM21/Ro52 accounted for the majority of relationships in Cluster 4. The remaining AAs; RNAP3, Ku, Fibrillarin and NOR90, were not present in sufficient numbers to determine any statistically significant outcome. We assessed those patients with CENP A/B and Topo1 expression, either singly or in combination who were in Cluster 4; RNAP3 and where numbers permitted, and the most common SSc associated AAs including TRIM21/Ro52 and Pm-Scl. In addition, we undertook a sub-analysis on those patients in Cluster 4 who were subsequently found to have U1RNP and we were able to compare these patients with a separate cohort of well-characterised MCTD patients within the ASCS cohort.

In line with the results, this discussion will be grouped according to the AAs tested; firstly the primary AAs; CENP, Topo1 and RNAP3 followed by TRIM21, PmScl and U1RNP.

Cluster 4 CENP, Topo1 and RNAP3

Although infrequent, these three primary AAs were represented in Cluster 4; however their presence was atypical, as demonstrated by single epitope expression (in CENP, RNAP3), lower staining intensity scores, or by co-expression with one or more AAs of equal or higher intensity staining. There were minimal differences between the expression of each of these AAs in Cluster 4 when compared with their expression in the cohort as a whole.

The increased frequency of male gender and oesophageal dysmotility observed in both the CENP and Topo1 Cluster 4 groups (as compared with expression of these AAs in the other clusters) is intriguing, but multinational studies are required to direct further interpretation.

Two further differences were noted in Cluster 4 Topo1 positive patients compared with Topo1 expression elsewhere. Those in Cluster 4 had reduced frequency of ILD

and increased PAH. Among those Cluster 4 Topo1 positive patients that had PAH, co-expression included Fibrillarin, CENP B, Pm75, and TRIM21/Ro52 with the former two AAs previously reported to be associated with PAH (19, 205).

Given the frequent co-occurrence of other AAs in these patients, it is likely that they may have an overlap variant of SSc. While not reaching significance, in Cluster 4 CENP patients (35.7% vs All CENP 15.5%, p= 0.069), that had ILD, Topo1 and TRIM21/Ro52 were co-expressed and again both of these AAs have been associated with ILD in this and other cohorts (22, 76, 282).

Coexistence of Topo1 and CENP

Topo1 and CENP have previously been thought to be mutually exclusive (129, 246, 324-327). However, with the advent of the newer diagnostic platforms, and the ability to test for multiple autoantibodies, this paradigm is shifting (324).

Among our 505 patients, 15 tested positive for CENP and Topo1. Perhaps surprisingly, these patients were dispersed through the Clusters identified in Chapter 3 and this no doubt reflects that in many cases one AA had the dominant staining pattern. The LIA used to detect these AA has been well-validated and we feel these findings reflect a true dual positive status rather than laboratory error. We used a single operator, single platform approach and testing by independent laboratories had excellent agreement (Topo1 κ=0.837 and CENP κ=0.887) with the results of the LIA (295-297). Understandably, genetic background, the type of cohort (population based or registry) and the choice of AA detection method will all have a bearing on both autoantibodies and clinical associations.

Overall this study found that the Topo1/CENP 'double positive' group had more in common with Topo1 patients than they did with CENP patients. On review, staining intensity in these dual positive patients was evenly distributed, with 8/15 have dominant Topo 1 AA expression and the remainder had dominant CENP A or B AA expression. In further detail, the 15 patients that were double CENP/Topo1 positive were compared with the 97 other Topo1 patients. There were two significant differences; double positive patients experienced Raynaud's younger and they all had sicca symptoms. In all other regards they were statistically clinically comparable with Topo1 patients.

In comparing single positive CENP patients with the 15 double positive patients, more differences were found in the Topo1/CENP group. There were more males, these patients had a younger age of disease onset, there was more diffuse disease, ILD was increased and systemic hypertension was decreased, they had less anal

incontinence and a higher Serum CK.

The EUSTAR cohort (n=4687) has also recently explored the clinical characteristics of ‘double positive’ patients and they found that although not statistically significant, their double positive patients, when compared with single positive Topo1 (control) patients, also experienced Raynaud’s at a slightly younger age. Overall, they concluded that their double positive patients were not ‘truly clinically different’ from their single positive Topo1 positive patients (324). Unlike our cohort, their double positive sera was more likely to come from female patients and there was a trend for their double positive patients to have more severe or prevalent involvement of the musculoskeletal system and pulmonary fibrosis (324) as compared with CENP positive patients.

Although our double positive patient cohort, like EUSTAR’s, had more in common with single positive Topo1 patients, the finding of more sicca in our double positive patients as compared with Topo 1 patients was interesting. A study by Avouac et al found that ‘*the principal cause of sicca syndrome in SSc appears to be glandular fibrosis, rather than SS lymphocytic sialadenitis’*, and was associated with CENP and limited scleroderma (328). This may provide some evidence of the effect of CENP on sicca in our cohort, although it may be that our double positives are more fibrotic compared with CENP patients and the sicca is the result of fibrosis alone and not the effect of CENP.

One last interesting finding was increased serum CK in our double positive patients compared with other CENP positive patients. This may relate to the presence of Topo1 as others have reported that among males, an increased serum CK was associated with Topo1 and diffuse disease (329).

A German study by Dick et al (327), asked the question if Topo1 and ACA are regarded as two separate clinical entities and patients express both AAs, do these patients have both diseases independently? On balance, SSc is more easily described as a spectrum of disease (45, 330) with many common clinical manifestations. Autoantibodies and their clinical associations seem to be variations of the same condition with the AA of the highest titre reflecting the dominant phenotype in our work and that of others (22). Autoantibodies may influence or reflect the clinical course but as yet they have not convincingly been shown to be pathogenic. It may also be that some AAs are dominant over others as a result of genetic or sex related reasons.

Due to the rarity of double positive patients, most studies lack statistical power and so our results and those of others must be interpreted with caution. In addition, different assays may have differing sensitivity and specificity profiles; IIF patterns may be wrongly interpreted or the pattern of an AA with a higher titre may mask the pattern of a different, weaker AA titre. Allowing for these technical concerns, the advent of new technologies means detection of double positive patients is likely to increase and multinational collaborations will allow for further investigation of this interesting cohort.

In conclusion, the double Topo1/CENP positive patients in this cohort are clinically more similar to Topo1 patients than CENP patients. It appears that they may have a more fibrotic phenotype while the presence of CENP may have a modulatory effect in regards to some clinical characteristics. Larger multinational studies will be required to see if these characteristics are carried across different SSc cohorts.

TRIM21/Ro52

In our cohort, TRIM21/Ro52 patients experienced a higher ESR compared with other patients and this may reflect a degree of overlap disease. In the German Network for Systemic Scleroderma Registry, TRIM21/Ro52 was associated with a higher ESR and Raynaud's phenomenon (81), and although we did not find a significant association with Raynaud's, all patients who were positive for TRIM21/Ro52 also experienced Raynaud's.

A higher sPAP, was observed in this monospecific TRIM21/Ro52 cohort but PAH was not increased and the difference in estimated systolic pulmonary artery pressure between the groups is not great. Prospective monitoring of these monospecific patients will help to determine whether the small differences in pulmonary pressures equates to an increase in the frequency of PAH.

Patients with monospecific TRIM 21/Ro52 in Cluster 4 had an older age of disease onset and this was also found to be the case in a multinational study (222) that included not only the ASIG patients, but also patients from the Canadian Scleroderma Research Group and the American Genetics versus Environment in Scleroderma Outcome Study (246). TRIM21/Ro52 is the most commonly co-expressed AA and its role in the ubiquitin process is an important one as well as its ability to regulate downstream signalling of various pattern recognition receptors such as the NF-κB, TGF-β and interferon response. Perhaps underlying senescence changes in cellular elements (331, 332) are involved in the pathogenesis of this abundantly expressed AA.

In the present study, a trend was observed between the presence of monospecific TRIM21/Ro52 and ILD (p= 0.09), while in a multinational cohort of SSc patients from Australia, Canada and the USA, ILD was the only clinical variable significantly associated with monospecific anti-TRIM21/Ro52 antibodies (282). A Norwegian study (using ANA Profile 5 Euroline Blot test kit; Euroimmun, Lubeck, Germany), found that the odds ratio for the presence of TRIM21/Ro52 AAs in lung fibrosis was 4.4 (95% CI 1.8-10.3) in MCTD patients (242). In our initial PCA, no association was found with ILD and TRIM21/Ro52, but this analysis was on a positive/negative basis and monospecific analyses were not done. In more recent studies, TRIM21/Ro52 appears in other autoimmune conditions with other AAs such as anti-aminoacyl transfer RNA synthetase antibodies and anti Jo-1, and these have been associated with ILD (333). Although there are still many questions regarding autoantibodies, it may be that different combinations of AAs are protective or act in synergy when combined with other AAs and so the clinical manifestations may vary from individual

to individual notwithstanding genetic background.

PmScl

PmScl monospecific patients experienced both Raynaud's and onset of non Raynaud's symptoms at a younger age than PmScl negative patients. Otherwise, they were statistically comparable to PmScl negative patients. Specifically, no differences were found for myositis, calcinosis, digital ulcers or gastrointestinal manifestations, although differences in all of these clinical manifestations have been reported in the past, generally in the presence of other co-expressed AAs (81, 200, 227, 334) (222) (335) (52). A Canadian led, multinational study (which includes data from this Australian cohort) investigating monospecific PmScl epitopes found that although it wasn't statistically significant, patients that were PM100 positive were younger at disease onset compared to PM75 and other AA subsets (222). In this study, there were insufficient numbers to interpret PmScl data when split into its epitopes.

Patients who are monospecific for PmScl are rare, and the Canadian led multinational study illustrates how trends observed in a single population can be further explored in larger cohorts. In this case, the trends we observed in our Australian cohort were reflected in the multinational study but there is little to suggest that the presence of monospecific PmScl can be used to predict clinical disease course.

Th/To

In the initial PCA analysis of Cluster 4 patients, Th/To was one of the AA which, along with TRIM21/Ro52, PmScl and the primary SSc AAs, had most influence in generating variability in Cluster 4. However, due to the small number of Th/To monospecific patients in this cluster (n=3) we were not able to explore any clinical associations. In addition, while the LIA detects AA to the hPop1 antigen (Ag) in the RNase MRP, recent work has suggested AAs to other more prevalent antigens in this complex (such as Rpp25 and Rpp38) (184). It will be interesting to explore clinical associations between the different Th/To Ag targets but as with other rarer SSc AAs, these questions will need to be explored in larger multinational cohorts.

U1RNP - SSc

Only 11 U1RNP positive patients were identified in Cluster 4 but 7/11 had high titre AA present and in 4 cases it was the only AA detected. We wished to explore how patients' positive for U1RNP fit within the SSc spectrum of disease.

Given there were a large number of AA negative sera in Cluster 4 and that most were ANA positive (many with speckled ANAs), we decided that it was important to test this Cluster, if sera were available, for a common AA that was not included on the SSc immunoblot. U1RNP patients are also included in the ASIG database and it seemed that this was the most logical AA to test for given the patients had a diagnosis of SSc and U1RNP is a known associated SSc AA. Had resources permitted, the entire 505 patients would have been tested, but as Clusters 1-3 and 5 were highly representative of their dominant Cluster AA and Cluster 4 was more heterogeneous, we decided it was more prudent to retest this Cluster for U1RNP.

Age demographics

U1RNP positive patients (with any disease classification) have consistently been reported in the literature as having a younger age of onset for both Raynaud's and the first non-Raynaud's symptom (22, 27, 218) and our data is consistent with this finding.

Male gender

It is unclear why there are more males represented in this U1RNP AA subgroup and Cluster 4 as compared with the SSc cohort as a whole, but perhaps it reflects that males are more likely to have atypical AA associations in SSc

C3 below normal, ever

Hypocomplementemia is a result of increased complement-component consumption in inflammatory conditions and was proposed by Hudson in 2007 as a marker of overlap disease in SSc (336). More recently, Esposito et al (337) found that reduced C3 and C4 are associated with some features of increased SSc disease activity (digital ulcers amongst others), in patients with overlap disease. Hypocomplementaemia and overlap disease association was also reported by Sturfelt and Truedsson who suggested that evidence of complement activation is related to the dysfunction of regulatory proteins and this could contribute to complement activation in the inflammatory process and subsequent vascular damage in SSc. They also stated that hypocomplementaemia is preferentially seen in patients with overlap disease features, especially SLE (338). An increased history of complement activation in the U1RNP positive Cluster 4 patients may reflect underlying overlap disease features.

Figure 4-9: Gangrene in SSc
https://consultqd.clevelandclinic.org/2014/1

Digital gangrene

Digital gangrene occurred more frequently in Cluster 4 U1RNP positive patients, although the presence of one patient with coexistent antiphospholipid syndrome may have biased results. It will again be useful to review these preliminary finding in larger, multinational cohorts.

Left ejection fraction volume

Although a difference in LVEF was reported between U1RNP positive and negative patients, the LVEF for both groups was well within normal range and therefore is unlikely to be of any clinical significance. Indeed, the 'normal' classification for LVEF is set at 55% to 65% (339) and LVEF ranges in this cohort mean LVEF was 60.42 % +/- 8.36 in the cohort as a whole.

Comparison of U1RNP SSc patients and U1RNP MCTD patients

Comparing the two subgroups of patients, *U1RNP SSc* and *U1RNP MCTD*, two important clinical differences have been revealed. Firstly, the MCTD group have milder vasculopathy, evident in less calcinosis and no digital gangrene and secondly, milder skin involvement. Otherwise, the two patient subgroups are statistically comparable for all other variables that are consistent with SSc. This suggests that U1RNP positive MCTD patients fit in the milder end of the SSc spectrum and patients classified as U1RNP positive SSc experience a more severe form of the disease.

Conclusion

Patients in Cluster 4 have definite SSc according to Medsger or ACR/EULAR criteria but lack, or have atypical expression of one of the three primary disease associated AAs. In this cohort, dual positivity of CENP and Topo1 was more closely associated with features of diffuse cutaneous rather than limited cutaneous features of SSc.

Sample sizes of monospecific AA expression were small but we were able to further investigate the clinical associations of monospecific TRIM21/Ro52 and PmScl expression and generally our findings or trends were consistent with those observed in other cohorts. Monospecific TRIM21/Ro52 patients developed Raynaud's phenomenon later in life, recorded a higher annual ESR and had a higher sPAP (although this was not associated with an increased frequency of overt PAH) while PmScl monospecific patients had a younger age of onset compared with tested ASCS cohort as a whole. These associations have also been assessed in larger cohorts but there is no indication at this time that monospecific staining of these AA in our cohort provided additional information that would change prognostication or disease management.

In addition, we tested selected patients in Cluster 4 for U1RNP and compared this cohort with a separate cohort in the ASCS with documented MCTD. We found that while these cohorts were essentially similar, SSc U1RNP positive patients had more severe skin involvement, vasculopathy and digital gangrene. Truly AA negative patients also represent an interesting and significant subgroup and will be explored further in the next chapter.

Cluster 4 patients represent a more heterogeneous SSc group and our explorative analyses suggest that further subgroups exist within this cohort. Multinational studies will be invaluable in characterising these patients further.

CHAPTER 5
AUTOANTIBODY NEGATIVE SYSTEMIC SCLEROSIS

Introduction

Autoantibodies (AA) are described as a central feature of SSc (13, 65, 340) and yet, in most cohorts, regardless of geographic location, there are a small percentage (0.2 – 5.8%) diagnosed with SSc who are not only AA negative, they are also anti-nuclear antibody (ANA) negative (44, 81, 122, 341) . There are a number of questions that follow in regards to this unusual serological finding;

i. Do these patients truly have SSc? And if so,
ii. What are the clinical manifestations that have led to this diagnosis?
iii. How have they been classified and/or stratified within the SSc spectrum?
iv. What is the prognostic outlook for these patients?
v. Do these patients form a distinct clinical subgroup?

This chapter reports on the clinical associations and demographic data of the AA / ANA negative patients in this cohort and also how these patients might be stratified within the SSc spectrum.

Methods

For a detailed explanation of the patient population, definitions of clinical characteristics, AA testing methodology and statistical analysis, see Chapter 2 Methods, pp. 65-77.

Results

Autoantibody Analysis

Among the 505 SSc patients in the Australian Scleroderma Cohort Study (ASCS) database who had sera available and were initially tested with the SSc immunoblot, 56 (11.08%) had negative results. These patients were identified for further testing and 19 (3.76%) were subsequently found to remain autoantibody negative on testing by ANA 5 line blot (which tests for U1RNP) and also ANA negative (by IIF as tested by regional laboratories and recorded in the ASIG database). These remaining 19 patients were retested by IIF and CIEP for Topo1, U1RNP, Sm, Jo1, PmScl, Ro, La and signal recognition protein. IIF pattern analysis did not indicate RNAP3 positivity. Twelve (2.37%) patients were confirmed AA / ANA negative by all methods.

Patient Demographic Analysis

There were 8 (66.6%) females and 4 (33.3%) males (female to male ratio 2:1) represented and according to the LeRoy et al (287) criteria, 5 (41.7%) had dcSSc and 7 were lcSSc (58.3%). Of the females, 2 had dcSSc and 6 had lcSSc and in the males, 3 had dcSSc and 1 had lcSSc (Table 5 -1).

Table 5-1 Summary gender and disease classification

		Disease Sub classification		
		Diffuse	Limited	Total
Gender	Female	2	6	8
	Male	3	1	4
Total		5	7	12

The data were normally distributed and a Mann Whitney U test revealed that the AA negative and AA positive groups were statistically comparable for age at Raynaud's onset, age at first non-Raynaud's symptom onset and disease duration (Table 5-2).

Table 5-2: Demographic analysis, AA/ANA neg vs AA pos

Demographic	Mean (years) ±SD	Total n	p
Age onset Raynaud's			
AA Negative	46.18 (17.48)	9/12	0.123
AA Positive	41.17 (15.57)	482/490	
Age onset first non Raynaud's symptom			
AA Negative	48.18 (13.06)	12/12	0.186
AA Positive	45.56 (14.08)	486/493	
Disease Duration (from onset non-Raynaud's)			
AA Negative	10.19 (12.02)	12/12	0.089
AA Positive	12.22 (10.01)	486/493	

Variability between the cohorts can be visualised in Figures 5-1 to 5-3. The median is noted as the horizontal line in each box and whisker plot. For each variable measured, the interquartile range (IQR) is used as a measure of variability and demonstrates the spread of data of where the central 50% of values fall from the median. As the IQR eliminates outliers, it best represents the dispersion of data. The median and interquartile range for age for AA negative and AA positive subgroups for each variable can be seen in Table 5-3.

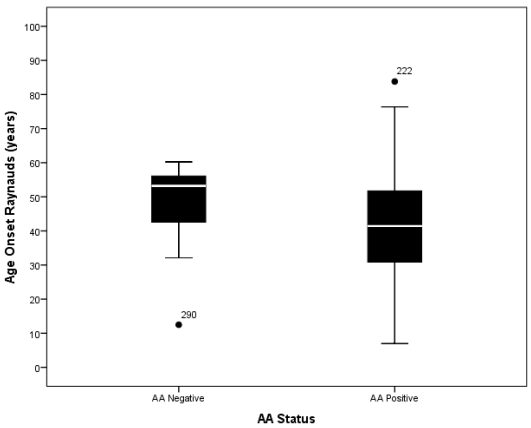


Figure 5-1: AA negative, Age onset Raynaud's

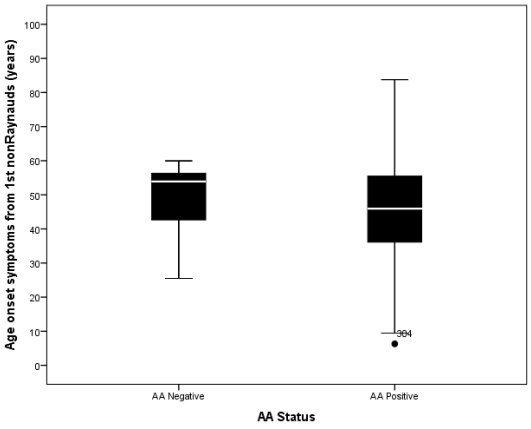


Figure 5-2: Onset first non-Raynaud's symptom

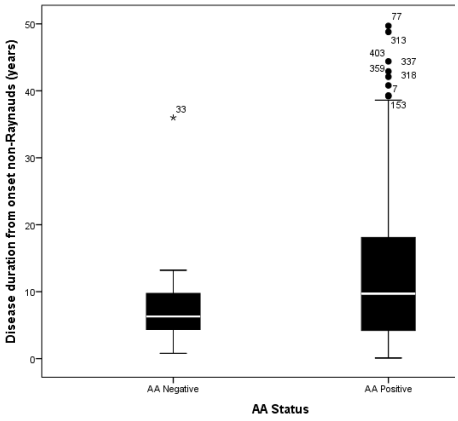


Figure 5-3: Disease duration from first non-Raynaud's symptom

Table 5-3: Demographic variability of AA negative and AA positive cohorts

	AA Status	Median	Interquartile Range
Age Onset Raynaud's (years)	Neg	53.56	37-52
	Positive	41.55	30-60
Age onset symptoms (non-Raynaud's) (years)	Neg	53.54	47-59
	Positive	46.06	36-56
Disease duration from onset non-Raynaud's (years)	Neg	6.10	2-11
	Positive	9.45	4-18

Clinical Characteristics

Ten of the twelve (83.3%) participants fulfilled the ACR (1980) criteria and 12/12 (100%) fulfilled the 2013 revised ACR/EULAR criteria. Demographic characteristics of AA negative patients are summarised in Table 5-4.

Table 5-4: Summary, clinical and demographical data, SSc AA negative patients

		Patient											
		53	66	163	345	472	522	638	802	807	854	953	1110
Disease Sub-classification	Diffuse	0	Yes	Yes	0	0	Yes	Yes	0	0	0	0	Yes
	Limited	Yes	0	0	Yes	Yes	0	0	Yes	Yes	Yes	Yes	0
Gender	Female	Yes	0	Yes	0	Yes	0	Yes	Yes	Yes	Yes	Yes	0
	Male	0	Yes	0	Yes	0	Yes	0	0	0	0	0	Yes
Age Onset Raynaud's (years)		32	53	0	54	60	58	12	53	Yes*	62	Yes*	0
Age		74	60	68	66	72	67	37	72	59	68	56	65
Age onset symptoms (non Raynaud's) (years)		32	53	50	54	60	58	25	54	46	59	50	61
Disease duration from onset non-Raynaud's (years)		36	1	12	6	6	3	6	13	8	4	2	1
ACR criteria filled	No	0	0	0	0	0	0	0	0	No	No	0	0
	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0	Yes	Yes
ACR/EULAR 2013 criteria	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ILD ever	No	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	0	Yes	0	0	Yes	0	0	0	0	0	0	0
PAH Status	No	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	0	0	0	0	Yes	0	0	0	0	0	0	0
EGC RBBB Ever 7 Reviews	No	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	Yes	0	0	0	Yes	0	0	Yes	0	0	0	0
Echo, pericardial effusion Ever	No	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	0	0	0	0	0	0	0	Yes	0	Yes	0	0
Highest Rodnan Score Ever 7 Reviews		5	38	13	4	11	30	27	8	Missing	Missing	3	42
Telangectasia Ever 7 Reviews	No	0	0	No	No	0	No	No	No	0	0	0	0
	Yes	Yes	Yes	0	0	Yes	0	0	0	Yes	Yes	Yes	Yes

* No age for Raynaud's onset was recorded in the ASIG database, however Raynaud's positivity was recorded

		Patient											
		53	66	163	345	472	522	638	802	807	854	953	1110
Calcinosis Ever 7 reviews	No	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	0	0	0	Yes	0	0	0	0	Yes	Yes	0	0
Dry mouth ever	No	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	Yes	0	0	Yes	Yes	0	Yes	Yes	Yes	0	Yes	0
Dry eyes ever	No	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	Yes	0	0	0	0	0	Yes	Yes	Yes	Yes	Yes	0
Reflux oesophagitis definite ever	Definite	Yes	Yes	0	0	Yes	0	Yes	Yes	Yes	Yes	Yes	Yes
	No	0	0	0	0	0	0	0	0	0	0	0	0
Synovitis definite ever 7 reviews	No	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	0	0	0	Yes	0	0	Yes	Yes	Yes	0	Yes	0
Digital ulcers ever 7 reviews	No	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	Yes	0	0	0	Yes	Yes	0	0	0	0	0	0
Systemic hypertension ever 7 reviews	No	0	0	No	0	0	0	0	No	0	0	0	0
	Yes	Yes	Yes	0	Yes	Yes	Yes	Yes	0	Yes	Yes	Yes	Yes
Nailfold capillary dilation ever 7 reviews	Yes	Yes	Yes	Missing	Missing	Yes	Missing	Yes	Missing	Yes	Missing	Yes	Yes
Tendon friction rubs ever 7 reviews	No	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	0	0	0	0	0	0	0	0	0	0	0	Yes
Joint contractures ever 7 reviews	No	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	0	Yes	Yes	Yes	0	0	0	0	0	0	Yes	Yes

Using the AA status (negative or positive) as the independent variable, a univariate analysis was carried out on each clinical characteristic (See Chapter 2, Methods pp. 65-66 for a complete list of all variables tested), to determine if any characteristics were associated with the AA negative status. Due to the small AA negative cohort, Fishers Exact test was used to determine significance and results must be interpreted with caution.

The clinical manifestations that are significantly associated with AA negative status were EGC right bundle branch block (p=0.025), telangiectasia (p=0.004), the presence of Raynaud's phenomenon (p=0.005), systemic hypertension (p=0.041) and male sex (p=0.048). There was one trend recognised; being the absence of digital ulcers (p=0.083) in AA negative patients (Table 5-5)

Table 5-5: Significant clinical associations and trends for SSc AA negative patients

	AA Negative n=12 (%)	AA Positive n=493 (%)	Fisher's Exact Test p
Raynaud's - Yes (%)	10/12 (83.3)	490/493(99.4)	0.005
Telangiectasia – Yes (%)	7/12 (58.3)	447/492 (90.9)	0.004
ECG-RBBB (Abnormal ever)	3/12 (25)	24/467 (5.1)	0.025
Systemic Hypertension –Yes (%)	10/12 (83.3)	259/493 (52.5)	0.041
Male sex (%)	4/12 (33.3)	58/493 (11.8)	0.048
Trends			
Absence of Digital Ulcers (%)	9/12 (75)	255/493 (51.7)	0.083

Highest Modified Rodnan Skin Score (mRSS)

We were also interested to assess the highest degree of skin involvement recorded in these patients as recognised by mRSS. Initial analysis showed that the AA positive group was unequally distributed (Levene's test (F=7.261, p= 0.007)) and a subsequent Kruskal-Wallis test (p=0.330) revealed the median scores were not statistically different (Figure 5-5).

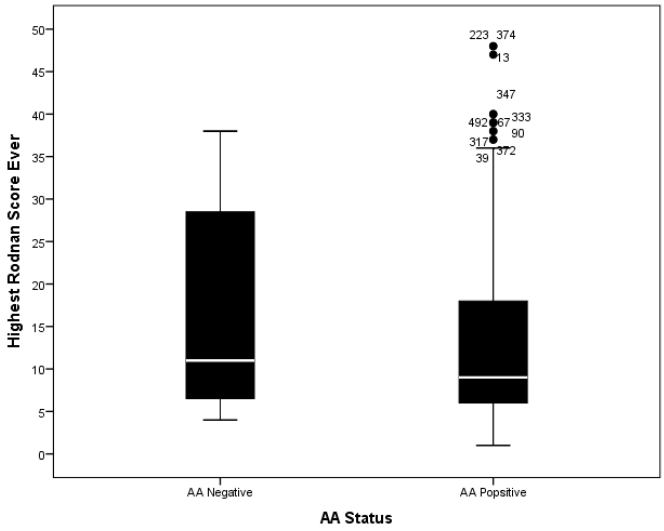


Figure 5-5: Data variation and median in AA negative and AA positive groups.

The median is represented on each box and whisker plot by the white horizontal line. The interquartile range (IQR), demonstrating the spread of data both within and between the two groups, can be visualised in Figure 5-5. The median for the AA negative group was 12 (IQR 5 – 32) and for the AA positive group the median was 9 (IQR 6 -18).

Malignancy

Three (25%) patients negative for AA on all testing had a history of malignancy (Table 5-7). Two patients had breast cancer and one patient had testicular cancer. There was no increased frequency of malignancy compared with the AA positive group (Fishers Exact test (p=0.185). In the remaining cohort (n= 493) tested by the ASCS, there were 61 patients that developed either a haematological or solid tumour with non-melanoma skin cancers excluded. Breast

cancer was the most common cancer experienced in the ASCS (19 AA positive patients, 2 AA negative patients).The AA/ANA negative male was the only one to have testicular cancer in the ASCS, although 3 AA positive males experienced prostate cancer.

Table 5-7: Summary of Malignancy in AA/ANA negative patients

		Gender	Disease Sub Classification	Date of Malignancy	Date 1st non-Raynaud's symptom	Malignancy Type
Blot & ANA Neg	Negative	Female	Limited	DEC 2009	JAN 1972	Breast
		Male	Diffuse	AUG 1985	MAR 2007	Testicular
		Female	Diffuse	JAN 1994	JAN 1996	Breast

Cluster Analysis

All AA negative patients were stratified into Cluster 4, 'Other' in the Principal Component Analysis (see Chapter 3, Figure 3-2, p.80).

Discussion

Among our cohort of 505, well-characterised patients with SSc, 12 (2.37%) patients were both AA and ANA negative on all testing. Given these small numbers, the clinical significance of our findings must be interpreted with caution. Nonetheless there were some interesting outcomes that can be compared to AA negative patients in other international cohorts.

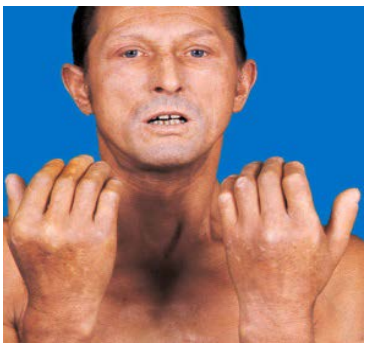


Figure 5-6: SSc male (source: Wolff, K et al. www.accessmedicine.com)

Male Gender

SSc has a female predominance ranging between 80 and 90% regardless of geographic location (302, 342). In our AA negative cohort, we observed a greater frequency of male patients (33.3%, p=0.048) as did a large US led study (44) (n=3249) where ANA negative males also formed a significant subgroup (41/ 208 or 19.7%, p=0.008). While males were represented in other AA, ANA and ENA negative studies (81, 341, 343), significance was not reached.

The effect of sex hormones may contribute to the finding of increased males in some studies. There is evidence that androgens decrease B cell maturation, reduce B cell synthesis of antibodies and suppress AA production in SLE (344), and in rheumatoid arthritis, as androgen levels decrease, Th2 responses and AA production increase (345). Testosterone also promotes a pro-inflammatory Th1/Th17 response that leads to fibrosis(344). It was interesting that three of the four male patients in the AA negative cohort had mRSS scores in the 75th percentile of all AA skin scores, 38, 30 and 42. All three were in the early stage of their disease

(≤3 years) and were classified as dcSSc. The remaining patient was enrolled 3 years after diagnosis and was classified with limited disease. Since enrolment his highest mRSS was 4, and any clinical details prior to enrolment are unavailable. A better understanding of the influence of sex hormones as well as epigenetic mechanisms and the influence of X and Y chromosomes on disease pathogenesis may elucidate differences in sex distribution in AA positive and negative patients.

Raynaud's Phenomenon

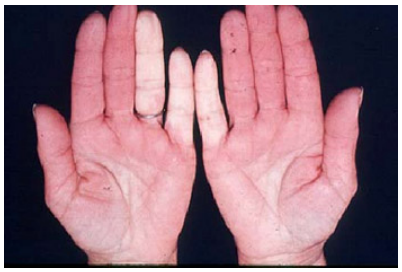


Figure 5-7: Raynaud's phenomenon in SSc.

Raynaud's phenomenon occurs in more than 90% of SSc patients and can precede SSc diagnosis, skin and visceral fibrosis and serum AAs by years or even decades (272). In this AA negative cohort, there was a lower incidence of Raynaud's phenomenon (83.3% vs 99.4% of AA positive patients). With the exception of a US led study (44), a lack of Raynaud's is a shared finding in other AA/ANA negative cohorts such as the Canadian (2/15, 13.3%), German (7/50, 14%) and EUSTAR (12/5378, 0.2%) studies. A critical issue in SSc is early diagnosis to prevent irreversible damage and 'red flags' such as Raynaud's, 'puffy hands', and AAs are vital early indicators of developing disease. Among those patients with ANA negative disease where Raynaud's is also absent, diagnosis is likely to be further delayed.

Digital Ulcers

Both the US and German led studies reported less digital ulcers in their cohorts (44, 81) and a trend towards less digital ulcers was also noted in this cohort which is an expected finding given the reduced presence of Raynaud's phenomenon.

Telangiectasia

Telangiectasiae are an important diagnostic indicator in SSc (142) and are a marker of a more severe vascular phenotype (346). In this cohort and the large US based cohort (44), telangiectasias are less frequently found in patients without circulating AAs or a detectable ANA. This would suggest a less vasculopathic form of disease (44).

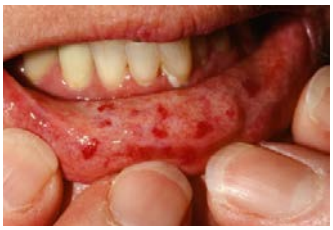


Figure 5-9: Telangiectasia in SSc.

Skin involvement

We did not detect any significant difference in the degree of skin involvement in our AA negative cohort, according to the highest mRSS recorded during each patient's involvement in the ASIG cohort. However, we recognise that these patients were not enrolled at the time of disease onset and so their peak skin involvement may have occurred prior to enrolment in this study. Inception cohorts will be invaluable to definitively determine if any differences in skin changes exist between ANA negative and positive patients. Other groups have also explored this question. Hudson et al reported a trend towards lower mRSS among AA negative patients and taken together with a lower prevalence of Raynaud's and telangiectasia, among the AA negative group they concluded that AA negative patients had milder disease (341). This result was mirrored somewhat by Hamaguchi et al who found that among AA negative patients in their Japanese cohort (5%; n= 10/203), there was milder skin involvement and only rarely were PAH, ILD and digital ulcers detected (122). In contrast, Schneeberger et al reported a trend towards a higher mRSS and shorter disease duration among AA negative EUSTAR patients (343).

Right Bundle Branch Block

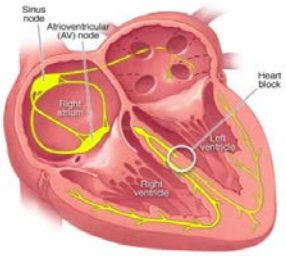


Figure 5-8: Bundle branch block

Diagnosis of a right bundle branch block (RBBB) is by electrocardiogram. It results from a block or delay in conduction of cardiac impulse through the right branch of the Bundle of His. Blood supply for this branch is from the septal vessels coming off the left anterior descending coronary artery (347). In our cohort 3/12 (25%) of patients experienced a RBBB result. The most common comorbidity of RBBB is systemic hypertension (347). Systemic hypertension was a significant cardinal feature of our AA negative patients with 83.3% experiencing this condition. In other cohorts systemic hypertension or RBBB are not reported. Only one patient in our AA negative cohort experienced pulmonary arterial hypertension (PAH) and this low prevalence seems to be the same as the German, Canadian, US and EUSTAR studies. Therefore we suspect that the higher prevalence of

RBBB may be a spurious finding perhaps influenced by the high prevalence of hypertension in our ANA negative patients. Hypertension is a common finding in the community and, in the absence of renal crisis, is not a disease specific feature of SSc. Its presence in our small cohort may again be a spurious finding and has not been reflected in other published data on ANA negative SSc.

Malignancy

Malignancy, while not reaching statistical significance in the AA negative subgroup, was present in 3/12 patients and included breast and testicular cancer. In 2/3 cases these diagnoses were remote form the diagnosis of malignancy. In our AA positive cohort, 61/493 (12.37%) patients had cancer and overall, cancer was found to be significantly associated with Cluster 4 (p=0.039). Other studies have found a close temporal association with SSc onset and a diagnosis of cancer, particularly with RNAP3 AAs (161, 164, 175) and also with breast cancer (348-350). It is noticeable that one AA negative patient was diagnosed with breast cancer 2 years prior to the SSc onset and Shah et al also found that AA negative patients had a close temporal relationship between SSc onset and cancer diagnosis and postulated that this group may express novel tumour antigens that are yet to be identified (173). In light of these findings it will be interesting to monitor disease associations in the Australian cohort as the duration of observation increases.

Many studies have investigated breast cancer in SSc and an increased risk or incidence was found in most (349, 351), although in a meta-analysis of 16 studies and over 7000 patients, the relationship with breast cancer and SSc was not conclusive (352). Atypical presentations of cancer have been found particularly in relation to breast cancer with paraneoplastic scleroderma and ANA negative patients. (353, 354). In the study by Schneeberger et al, four of the seven patients for whom detailed information was available, had a malignancy (two breast, one multiple myeloma and one bladder carcinoma) and a fifth patient who died three years after his initial visit may have had a malignancy but cause of death was not recorded (343). Despite not finding a statistically increased risk of malignancy in their AA negative cohort, Schneeberger et al still conclude that a paraneoplastic syndrome should be considered as a differential diagnosis in AA negative and Raynaud's negative patients (343). In other SSc cohorts where studies on AA negative patients have been conducted (US (44)and Canadian (341)), malignancy information was not available.

While any association between malignancy and AA negative SSc remains uncertain, it was not evident in our cohort. However, careful screening for malignancy in patients with any form of atypical presentation, including the absence of detectable AAs seems prudent.

Limited survival data is available on AA negative patients and at the time data for this study were censored (7 June 2013), all patients were still alive, so no survival analysis was possible. Only the Salazar et al study has reported on survival and they did not find a survival difference between AA negative and AA positive patients (44). (Figure 5-12). It will be interesting to see if this finding is reflected in other cohorts as further survival data becomes available.

Conclusion

Among 505 patients with confirmed SSc, only 12 patients were AA negative on all available testing. Ten of the twelve patients fulfilled the 1980 ACR criteria and retrospectively 12/12 fulfiled the 2013 ACR/EULAR criteria. We reviewed their clinical characteristics and all had features entirely consistent with clinical disease and in 5 patients skin involvement was consistent with diffuse disease. Further research in large multinational collaborations is required to stratify these patients particularly in regards to prognosis and survival.

Our cohort is clinically similar to other published AA negative cohorts. These AA negative patients have fibrotic features of SSc and are less likely to have the SSc associated vasculopathy characterised by Raynaud's phenomenon, telangiectasias, and to a lesser extent, digital ulcers. These findings and are in accordance with many of the findings of Hudson et al (341) and Salazar et al (44). Finally, in our cohort AA negative patients, for reasons yet to be elucidated, are more commonly male.

CHAPTER 6
COMPARISON OF METHODS

Introduction

Interpretation and analysis of autoantibodies in this scleroderma cohort have been undertaken using immunoblot technology. The SSc Euroimmun line blot has been validated in a number of cohorts and has good agreement with traditional methods of indirect immunofluorescence (IIF), enzyme linked immunosorbent assay (ELISA) and immunoprecipitation (IP) (144, 196, 355). Line immunoassays (LIA) are reported to be a faster and more practical method of extractable anti-nuclear (ENA) antigen detection (106, 196). For uniformity, testing was undertaken by a single operator at a single centre and the LIA analysis had the added benefit of testing for other rarer, SSc associated AAs that are not generally available in commerical laboratories.

Different methodologies will have differing sensitivities and specificities, even when targeting the same autoantigen Furthermore, while most AAs in the setting of SSc are considered to be constant, there is evidence that some, such as RNAP3, may fluctuate during the disease course (154). Particular factors to be considered when comparing two or more different testing methods include:

i) Epitope/vector expression – type of assay
ii) Diagnostic accuracy and reliability - validated in a variety of cohorts with different genetic and ethnic backgrounds
iii) Sensitivity and specificity
iv) Multiple autoantibody (AA) positivity
v) Expertise in interpreting results
vi) Clinical associations/prognoses with a particular AA or combination of AAs

A more detailed explanation of the Euroimmun line blot is detailed in Chapter 2 and Chapter 1 (pp. 14-17) includes a discussion of the different AA testing platforms available. In general terms, initial screening for ANAs are carried out using IIF on HEp-2 cells followed by a more specific method using a variety of platforms. Greater than 100 antigens can be detected using IIF and it is considered by some the ‘gold standard’ for ANA detection (356), although interpretation of rarer profiles and mixed patterns requires considerable expertise. Newer platforms such as the LIA have the ability to test large numbers of AAs using a commercially viable assay; however, there are concerns that denaturing of proteins may change the conformation of epitopes. An advantage of a platform such as the immunoblot is that there is

an increasing recognition that multiple positive AAs can be detected in a single patient (67). These results may not have been available in older assays which are perhaps more specific but may lack in sensitivity. However, it is uncertain whether increased sensitivity aids in clinical assessment in practical terms.

We were interested to compare the reliability of the Euroimmun LIA in this well characterised Australian population with other commercially available assays. Where there was discrepancy between testing, we were also interested to look at the individual results more closely and the clinical associations of these patients to see whether the test result might support the patient's disease sub classification. This chapter will compare results of the (Euroimmun) Line Immunoassay (LIA), with results obtained by independent laboratories in the Australian Scleroderma Cohort Study (ASCS).

Comparison of results

There were 505 patients in the SSc cohort and an additional 21 patients that were classified with Mixed Connective Tissue Disease (MCTD). In addition to the Euroimmun LIA, the results of independent commercial laboratory testing for ANA staining and AAs to CENP, Topo1 and RNAP3 were available for the majority of patients. Other rarer AAs were less frequently tested and for the purposes of this analysis, we limited comparisons to these three major AAs, shown to be the most clinically significant in our earlier work. All commercially available immunology laboratories in Australia participate in a Quality Assurance Program which provides feedback on the accuracy of AA testing. In Australia, the initial screening for ANA is undertaken with IIF using HEp-2 cells. Identification of CENP AA was by their characteristic IIF staining pattern. Further characterisations were generally undertaken by ENA with a precipitating antigen for Topo1 and where available, RNAP3 testing was also performed by ELISA. At the time of data census (7 June 2013), the majority of regional and central laboratories in Australia were using ELISA and IP for ENA testing, although with the advent of the newer diagnostic platforms, there is now greater variation of methods. Results from independent laboratories are referred to as Australian Scleroderma Interest Group (ASIG) data. LIA data was complete for all patients in this cohort.

Tables 6-1 to 6-4 are a summary of the results from ASIG independent laboratories for SSc (n=505) and include the supplementary RNP (MCTD) data (n=21) as tested by ASIG independent laboratories.

Table 6-3: Summary ASIG ANA by IIF

		Frequency	Percent
	No	30	5.7
	Yes	496	94.3
	Total	526	100.0

Table 6-4: Summary ASIG IIF Centromere

		Frequency	Percent
	No	310	58.9
	Yes	212	40.3
	Total	522	99.2
Missing		4	.8
Total		526	100.0

Table 6-5: Summary ASIG ENA Topo1

		Frequency	Percent
	No	433	82.3
	Yes	87	16.5
	Total	520	98.9
Missing		6	1.1
Total		526	100.0

Table 6-6: Summary ASIG RNAP3 (ELISA)

		Frequency	Percent
	N/A*	152	28.9
	No	301	57.2
	Yes	53	10.1
	Total	506	96.2
Missing		20	3.8
Total		526	100.0

* NOTE: RNAP3 was physician requested and so not all patients were tested for this AA.

Statistical analysis

For the purpose of comparing interrater reliability Cohen's kappa (κ) statistic, a measure of agreement between categorical variables, was used. A kappa of 1 indicates perfect agreement, whereas a kappa of 0 indicates agreement equivalent to chance. Further information on the κ statistic is found in Chapter 2, Methods (p.76).

Results

CENP

The results for the CENP immunoblot data were complete with 4 patients missing from the ASIG Centromere IIF results. Therefore, 99.2% (501/505) of data were utilised in Cohen's κ analysis. The LIA and ASIG data were in agreement on 206 (90.4%) positive results and 267 (97.8%) negative results. Cohen's κ analysis determined there was excellent or very good

agreement (κ = 0.887) between the two methods according to Landis & Koch (295), Altman (296) and Fleiss (297).

Discordant results are summarised in Table 6-5 with the majority of discordance being accounted for by immunoblot CENP positive and ASIG IIF centromere negative results.

Table 6-5: Comparison CENP LIA and ASIG IIF centromere independent laboratory results (% agreement)

		Blot CENP		
		Negative	Positive	Total
ASIG IIF Centromere	Negative	267 (97.8%)	22	289
	Positive	6	206 (90.4%)	212
Total		273	228	501

A summary of the 22 blot positive/ASIG IIF negative patients can be seen in Table 6-6.

The most useful way to interpret discordance between IIF Centromere and LIA CENP (Table 6-6), is to compare both ASIG AA testing and IIF staining patterns with the LIA intensity score. In most instances where LIA intensity scores are low, there are other LIA AAs with a higher intensity score and most often, the IIF staining or ASIG AA is concordant with the higher LIA intensity score. Six patients (5 LcSSc, 1 dcSSc) with a strongly positive CENP LIA ('+++') had a positive ANA on IIF from ASIG testing but IIF CENP was not detected. Five of these patients were designated lcSSc, which is supported by the Euorimmun CENP positive profile. One patient with diffuse cutaneous disease also had a strongly positive CENP but in addition, there was moderate staining for RNAP3 on the immunoblot. Clinical features of these 6 patients include ILD (4 pts), PAH (1pt), calcinosis (5 pts), sicca (6 pts), reflux (6 pts), oesophageal dysmotility (4 pts), digital ulcers (3 pts), Raynaud's (6 pts), systemic hypertension (3 pts), nailfold abnormalities (5 pts, 1 missing data), joint contractures (3 pts) and telangiectasia (6 pts).

Table 6-6: Summary blot CENP positive, ASIG IIF Centromere negative

Number patients in group	Classification	ANA ASIG	Highest mRSS	IIF Centromere	ASIG Results	CENP Blot Staining Intensity	Other Immunoblot staining
2	1 pt Limited 1 pt Diffuse	Negative	2 10	Negative	No IIF or ASIG AA ANCA&MPO	'+++' '+'	TRIM21/Ro52'++' § and PM75'+' None
6	Limited	Positive	6 - 23	Negative	1pt Topo1/Nucleolar 2pts Topo1/speckled 1pt homogeneous 1pt nucleolar/speckled 1 pt nucleolar	'+'	1 pt Topo1 '+++' and PM75 '++' 2 pts Topo1'+++', 1 pt Topo1, RNAP3 '+' TRIM21/Ro52 '++' and PM75 '++' 1 pt RNAP3'+++' 1 pt Th/To '+++'
3	Diffuse	Positive	10 - 27	Negative	3 pts Topo1	'+'	2 pts Topo1 '+++' & PM75 '+++' or '++' 1 pt Topo1 '+'
4	Limited	Positive	3 - 31	Negative	2pts Topo 1 pt RNAP3 1 pt nucleolar/speckled	'++'	Same 2 pts Topo1 '+++' Same 1 pt RNAP3 '+' 1pt TRIM21/Ro52 '+++' and PM'75 '++'
1	Diffuse	Positive	14	Negative	Topo1	'++'	Topo1 '+++'
5	Limited	Positive	2 - 23	Negative	2 pts homogeneous 2 pts speckled/homogeneous 1 pt nucleolar	'+++'	1 pt Th/To '+++' 1 pt NOR 90 '+++' All pts TRIM21/Ro52 varying between '+' '++' '+++'
1	Diffuse	Positive	14	Negative	Homogeneous	'+++'	RNAP3 '++'

§ Not tested in ASIG Laboratories

There were 6 patients that were ANA IIF centromere positive but LIA CENP negative. The AA profiles of these patients were reviewed. One patient was LIA positive for Ku ('++') and one was LIA positive for NOR90 ('+'). Upon retesting for U1RNP, 1 patient was LIA positive for this AA, with the remaining 3 patients being LIA negative. Clinical features of these patients included: telangiectasia (5 pts), calcinosis (3 pts), sicca (5 pts), reflux (4 pts), digital ulcers (3 pts), Raynaud's (6 pts), systemic hypertension (5 pts), nailfold abnormalities (5 pts).

Topo1

There were 6 (1.2%) patients missing ASIG Topo1 results therefore Cohen's κ analysis was performed on 499 (98.8%) patients. Of these patients, 112 were Topo1 immunoblot positive and 86 were ASIG Topo1 positive. The LIA and ASIG data were in agreement on 85 (76.6%) positive results and 387 (99.7%) negative results. Cohen's κ coefficient of 0.837 signifies excellent agreement based on Landis and Koch's (295) measurement of observer agreement for categorical data, and very good agreement according to Altman's (296) and Fleiss' (297) assessment.

Discordant results are summarised in Table 6-7 with the majority of discordance being accounted for by immunoblot Topo1 positive and ASIG Topo1 negative results.

Table 6-7: Comparison Topo1 LIA and ASIG ENA independent laboratory results. (% agreement)

		Blot Topo1		
		Negative	Positive	Total
ASIG Topo1	Negative	387 (99.7%)	26	413
	Positive	1	85 (76.6%)	86
	Total	388	111	499

A summary of the 26 blot Topo1 positive/ASIG ENA Topo1 negative patients can be seen in Table 6-8. To aid in interpretation, patients were classified in a number of different groups. It can be seen that among the 3/26 patients who were IIF negative all had been classified as having limited disease, even though some had a mRSS more frequently seen in diffuse disease. It was interesting to observe that despite negative ANA testing, the Topo1 results on immunoblot were of moderate or intense staining. There were 8/26 patients classified as limited and 1/26 classified as diffuse who had a weak positive ('+') blot staining intensity. Further analysis of the limited patients revealed that four were strongly immunoblot positive for CENP and one was weakly positive for RNAP3 with these results agreeing with ASIG testing. In patients that were classified as limited (6/26) or diffuse (1/26) with a positive ('++') blot intensity, the results were mixed. One patient was positive for CENP in both blot and ASIG and two patients had a nucleolar/speckled or speckled pattern. The remaining three patients had a homogeneous pattern. Finally, in the 2/26 patients with limited disease and 5/26 patients with diffuse disease all had strong ('+++') blot intensities. ASIG IIF results showed that 4 patients had a homogeneous pattern; one had a speckled pattern, one a speckled/homogeneous pattern and one a nucleolar/homogeneous pattern. Clinically these patients showed strong features that are associated with Topo1 (19, 357), such as ILD (11/26 or 42.3%), digital ulcers (12/26 or 46.2%) and joint contractures (13/26 or 50%).

Table 6-8: Summary, Blot positive Topo1 and ASIG Topo1 ENA negative results

Number patients in group	Classification	ANA # ASIG	Highest mRSS ‡	Topo1 ASIG ENA/IP	ASIG Results	Topo1 Blot Staining Intensity	Other Immunoblot staining
3	Limited	Negative	3 - 23	Negative	1 pt APL ϕ 1 pt ANCA § and MPO ¶ 1 pt Nothing detected	'+++' '++' '+++'	- - TRIM21/Ro52 '+++'
8	Limited	Positive	5 - 15	Negative	4 pts Centromere 1 pt RNAP3† (homogeneous) 1 pt homogeneous 1 nucleolar 1 speckled	'+'	Same 4 pts also CENP '+++' Same 1pt RNAP3 '+' Other AAs present PM75 '+++' or '++' or '+' TRIM21/Ro52 '+++' or '++'
1	Diffuse	Positive	8	Negative	Homogeneous	'+'	-
6	Limited	Positive	3 - 10	Negative	3 pts homogeneous 1 pt centromere 1 pt speckled 1 pt nucleolar/speckled	'++'	1 pt PM100 '++' Same 1 pt CENP '+++' and TRIM21/Ro52 '+++' and NOR90 '+'
1	Diffuse	Positive	12	Negative	Nucleolar and homogeneous	'++'	-
2	Limited	Positive	6 - 20	Negative	1 pt speckled/homogeneous 1 pt homogeneous	'+++'	1 pt PM75 '+' & PM100 '+'
5	Diffuse	Positive	4 - 35	Negative	1 pt homogeneous/nucleolar 1 pt speckled 3 pts homogeneous	'+++'	2 pts TRIM21/Ro52 '+++' 1 pt TRIM21/Ro52 '+'

Anti-nuclear antibody, ‡ Modified Rodnan Skin Score, ϕ Anti-phospholipid, § Anti-neutrophil cytoplasmic antibodies, Myeloperoxidase, † anti-RNA Polymerase III

There was one patient with limited disease (highest mRSS, 2), that was ASIG Topo1 ENA positive, ANA (IIF) and blot negative. Clinically this patient experienced Raynaud's, nailfold capillary dilatation, telangiectasia, reflux, digital ulcers, tendon friction rubs and joint contractures.

RNAP3

Data for Immunoblot RNAP3 are complete with 81 positive and 424 negative results. The ASIG data base shows that there were 53 positive and 290 negative results with 145 patients not tested (not routinely done) and 17 missing data. The Cohen's κ analysis excluded both missing data and patients that were not tested, therefore 343 (67.9%) of the patients were included in the Cohen's κ analysis. The LIA and ASIG data were in agreement on 48 (73.8%) positive results and 273 (98.2%) negative results (Table 6-9).

Table 6-9: Comparison LIA RNAP3 and ASIG ELISA RNAP3 results (% agreement)

		Blot RNAP3		
		Negative	Positive	Total
RNAP3 ASIG	Negative	273 (98.2%)	17	290
	Positive	5	48 (73.8%)	53
Total		278	65	343

Cohen's κ analysis (κ = .775) determined a range of agreement results from substantial (Landis and Koch, (295)), to very good (Fleiss (297)) to good (Altman, (296)).

Discordant results are summarised in Table 6-10 with the majority of discordance being accounted for by LIA RNAP3 positive and ASIG RNAP3 negative results.

Table 6-10: Summary blot RNAP3 positive, ASIG ELISA RNAP3 negative

Number patients in group	Classification	ANA ASIG	Highest mRSS	ASIG Results	RNAP3 ASIG	RNAP3 Blot Staining Intensity	Other Immunoblot staining
5	Limited	Positive	2 - 23	5 pts IIF Centromere	Negative	'+'	5 pts CENP '+++'
1	Diffuse	Positive	18	Nucleolar/speckled	Negative	'+'	PM75 '+'
5	Limited	Positive	3 - 18	2 pts IIF Centromere 2 pt speckled nucleolar 1 pt speckled	Negative	'++'	Same 2 pts CENP '+++' 2 pts also TRIM21/Ro52 '+++' or '++'
3	Limited	Positive	6 - 12	1 pt IIF Centromere 2 pts speckled	Negative	'+++'	Same 1 pt CENP '+++'
3	Diffuse	Positive	10 – 37	1 pt nucleolar 2 pts speckled	Negative	'+++'	2 pts also TRIM21/Ro52 '+++' or '+'

As previously described, RNAP3 LIA AA intensity scores should be compared with IIF patterns as well as RNAP3 ELISA. At the time sera were analysed in both central and regional laboratories, many would not have had RNAP3 ELISA available as a routine test. The higher LIA intensity scores are concordant with ASIG IIF results and clinical presentation.

Table 6-11 summarises discordant results for ASIG positive and LIA negative results

Table 6-11: Summary RNAP3 ASIG positive blot RNAP3 negative

Number patients in group	Classification	RNAP3 Blot Result	Highest mRSS	ASIG Results	ASIG ANA pattern	Other Immunoblot staining
2	Limited	Negative	7 - 11	RNAP3	Speckled	1 pt TRIM21/Ro52 '+++' 1 pt LIA blot negative
1	Limited	Negative	2	RNAP3	Speckled/ Nucleolar	LIA blot negative
2	Limited	Negative	9	2 pts IIF Centromere & RNAP3	Centromere	2 pts LIA CENP '+++'

The clinical characteristics of the patients in Table 6-11 are; ILD (2 pts), PAH (1pt), calcinosis (3 pts), sicca (5 pts), reflux (4 pts), synovitis (3 pts), digital ulcers (4 pts), Raynaud's, (5 pts), anal incontinence (4 pts), systemic hypertension (4 pts), nailfold abnormalities, (5 pts) and joint contractures (2 pts).

Discussion

The Euroimmin Scleroderma LIA is a validated, commercially available assay for the detection of multiple SSc associated AAs. Newer diagnostic technologies such as the immunoblot are increasingly used in commercial laboratory settings, but in the past, the majority of AA testing for scleroderma was performed using IIF with HEp-2 cells followed by ELISA for assessment of ENA, including RNAP3. In Australia, the majority of testing through commercial laboratories at the time of data censorship (July 2013) was performed using these methods and so we were interested to compare our LIA findings with the data obtained by ASIG from regional laboratories. It is important to recognise that the results recorded in the ASCS database were unlikely to be obtained from sera taken at the same time as the stored sera utilised for the Euroimmun. While this does not have relevance for the many AAs that are stable over time, there is some evidence that Topo1 and RNAP3 levels do fluctuate and so this may have affected interpretation of results.

For this validation study, we limited comparisons to the three primary disease associated AAs as the ASCS data for Topo1 and CENP/Centromere were largely complete and almost 70% of data were available for RNAP3, allowing for a good comparative study.

The Cohen's κ analyses for each AA showed that there was good to excellent agreement according to Landis & Koch, Altman and Fleiss for CENP and Topo 1 while there was substantial to very good agreement for RNAP3. Ideally, concordance should be complete but different testing platforms may not identity the same clinical cohorts, even if they are testing for the same AA. On closer review of discordant results, we found that IIF patterns on HEp-2 cells often had a stronger concordance with the LIA blot results rather than ENA or ELISA results. For example a patient positive for RNAP3 by LIA might have a speckled ANA by IIF even though ELISA for RNAP3 was negative. In addition, where apparent discordance occurred due to a positive LIA and negative ELISA, the LIA would also frequently demonstrate additional dominant staining of another AA identified through testing from regional laboratories. For example, 4 patients who were LIA blot positive for Topo1 with a staining intensity of '+', initially appeared discordant with ASIG results showing IIF centromere, but on further investigation on a case by case basis, these patients were also found to be strongly LIA blot positive for CENP '+++'.

While our cohort had insufficient numbers to compare testing platforms for the remaining AA on the LIA, others have explored validity of different methods with these AA. Some rare AAs are difficult to test for due to the technical complexity and interpretation of the IP assay and so and effective LIA for these AA allows testing to extend beyond the research setting. An

example of this is anti-U3-RNP/ Fibrillarin testing. Peterson et al compared the Euroimmun LIA with an IP assay and found that anti-U3-RNP/Fibrillarin IP and LIA results concurred in 98.9 % of patients, yielding a κ coefficient 0.966 (196) and had good clinical agreement. They concluded that the LIA is an acceptable and attractive alternative to IP for anti-U3-RNP detection. The same or similar Euroimmun LIA was used in an Italian (200), Singaporean (Chinese, Malay, Indian) (144) German (81) and Korean (358) population. Kappa analysis for the Singaporean cohort between the LIA and ELISA was evaluated by individual AA and ranged from κ=0.83 (CENP A), κ=0.96 (CENP B), κ= 0.97 (Topo1) to κ=1.0 (Pm-Scl) and evaluation between LIA and IIF for CENP A and CENP B was κ=0.81 and κ=0.77 respectively. This study also found good clinical agreement when compared with published data from other cohorts (144). The Italian study compared sensitivity and specificity and again found that their results were similar to those found in other studies and concluded that the LIA was 'a more rapid and more practical method than IP assays' (200).

Mierau et al assessed (81) the frequency of disease-associated and other nuclear AA in the German Network for systemic scleroderma utilising a variety of methods including LIA. They used a different AA profile (Euroimmun ANA Profile 3) to that tested in our cohort. Some AAs such as Ku, Th-To, Fibrillarin, RNAP3 NOR90 were tested by other methods and PDGFR was not tested at all. For those AAs that were in common with the LIA SSc (Nucleoli) profile (TRIM21/Ro52, Scl-70 [Topo1], PmScl, CENP B), clinical associations were similar to other published cohorts (81). The Korean study (n=948 SARDs but included only n=25 SSc) used the same ANA Profile 3 as the German study but the results were in contrast to both the German and other published results yielding a κ=0.573 for agreement between IIF and the LIA for SSc AAs and clinical diagnoses. The authors stated a potential limitation of the study were the clinical diagnoses that were obtained from medical records which may be subject to bias since diagnostic errors and the effect of past and current treatments could not be taken into account (358). Again, this highlights the issue that using different testing platforms may not identity the same clinical cohorts, even if they are testing for the same AA.

Overall, there is substantial agreement between the Immunoblot and the other methods used by the ASIG independent laboratories when comparing RNAP3, CENP and Topo1 assays. Other published series have reported clinical associations when LIA is used to detect the remaining AAs to be similar to AA detected by other methods.

The Immunoblot is more sensitive to AA detection with the added advantage of multiple AA recognition, although the clinical interpretation of multiple AAs requires further consideration. It allows for an individual patient profile by testing for rare AAs that can be difficult to obtain by

other methods. This initial work has shown that the LIA has a role in AA sub classification and our comparison with other testing platforms can provide reassurance that extrapolation to other testing platforms such as ELISA, IP and IIF as long as the AA with the highest titre AA is considered.

CHAPTER 7
CONCLUSIONS AND FUTURE RESEARCH

Conclusion

To conclude this thesis a final summary synthesising results and confirming the testing of hypotheses is presented below.

Principal Component Analysis

Recent advances in diagnostic technologies have allowed commercial laboratories to offer a wider array of AA testing for scleroderma and other disorders. Interpreting the clinical relevance of a positive result from these assays can be challenging, not only because of differing detection methods, but also because multiple AAs may be detected. This thesis has explored AA expression in scleroderma and uses mathematical modelling to aid interpretation of complex results.

The Principal components analysis (PCA) provided a novel means to stratify scleroderma patients according to the presence and intensity of a variety of scleroderma AAs. This method reduced confounding when more than one AA was detected in patient sera. Multiple AA expressions were common in this cohort, with an equal number of patients expressing either a single or multiple AAs.

The AA with the highest titre (the dominant AA) determined individual patient cluster allocation in the PCA and also correlated with meaningful disease associations. Patients with dominant primary AAs (CENP, Topo1, RNAP3) made up 4/5 identified clusters and demonstrated clinical features that are most commonly recognised with these AAs, limited and diffuse disease. Interestingly, RNAP3 separated into two small, separate clusters depending on the intensity of staining. In our analyses there was a trend towards higher titres occurring earlier in disease and it would be interesting to follow a larger cohort of RNAP3 patients prospectively for changes in titre over time and disease activity.

Fine Specificities

The PCA analysis also identified a group of patients (Cluster 4) who did not have one of the SSc primary AAs as their dominant AA, or had atypical co-expression of other AAs. This group comprised a significant component of the tested cohort (142/505 patients) and their diverse AA profile may represent a challenge to the treating clinician looking for confirmation of a clinical diagnosis. For the most part, these patients did not have one of the three primary AAs and if they did, it was of a lower staining intensity and often present in combination with other SSc

associated AAs. Autoantibodies to CENP, Topo1 (including double positive (CENP/Topo1) patients), TRIM21/Ro52, PmScl and Th/To were found to have the most substantial influence on variance in this Cluster. In addition, supplementary testing of selected patients demonstrated U1RNP in 11 Cluster 4 patients, even though their clinical presentation was most consistent with SSc rather than MCTD or overlap disease.

Positive associations in Cluster 4 as a whole included male gender, a history of malignancy and smoking. Negative associations included gender bias and joint contractures. We explored this cluster further according to their varied AA profiles to see if further information could be obtained within this subset. We first compared Topo1 Cluster 4 patients with those positive for Topo1in the entire cohort and repeated a similar analysis for CENP Cluster 4 patients. Some minor differences were noted in these groups, particularly an increased frequency of PAH and reduced ILD in the Topo1 Cluster 4 group, which again demonstrates disease associations are generally most pronounced with the dominant AA.

Co-expression of CENP and Topo1 was rare and these two AA have previously been thought to be mutually exclusive. Among our 505 patients, 15 patients co-expressed Topo1 and CENP and were dispersed among Clusters 3-5, usually reflecting the dominant AA staining intensity. The LIA used to detect these AAs has been well-validated and we feel these findings reflect a true dual positive status rather than laboratory error. We used a single operator, single platform approach and testing by independent laboratories had excellent agreement (Topo1 κ=0.837 and CENP κ=0.887) with the results of the LIA. Overall this study found that the Topo1/CENP 'double positive' group had more in common with Topo1 patients than they did with CENP patients.

Initial investigation of the less disease specific and/or rarer AA in our cohort revealed only a few specific disease associations. In many cases, numbers of these AAs were small and the ASCS data has been used in Tri-Nation studies to explore clinical associations of these AAs in a larger multinational cohort (222, 241). We further hypothesized that the dominant AA would have the greatest association with the clinical state. We were therefore interested to explore the clinical associations of those patients in Cluster 4 who were monospecific for scleroderma AA other than CENP, RNAP3 and Topo1. Sample size meant that meaningful analysis was only possible for monospecific PmScl, TRIM21/Ro52 and the presence of U1RNP.

Monospecific TRIM21/Ro52 had features of inflammation, myocardial involvement and a tendency towards ILD that was not evident in the larger cohort.

PmScl was rarely expressed monospecifically as either single or both epitopes. Monospecific

patients experienced Raynaud's and disease onset at a younger age than other SSc patients.

U1 RNP SSc positive patients were more likely to be male, have an earlier disease onset, have a history of reduced C3 and develop digital gangrene than the remainder of the cohort. In addition U1RNP SSc positive patients and U1RNP MCTD patients were marginally different with U1RNP MCTD patients having a milder phenotype with less vasculopathy and skin involvement. MCTD patients are seen as being within the SSc spectrum of disease. U1RNP positive SSc patients can develop serious vascular manifestations (digital gangrene), therefore we believe that this AA should be included in SSc testing panels.

This study demonstrated that where typical AA testing does not find one of the primary SSc AAs, then sera should be sent to a reference laboratory for further analysis. More effective analyses of rarer AAs will require multinational collaborations to explore clinical associations, genetic and environmental influences.

Autoantibody Negative Patients

SSc patients that are both AA and ANA negative are very rare and in this cohort there were only 12/505 patients (2.38%), with all fulfilling the 2013 ACR/EULAR criteria. These patients emphasize the need for classification criteria for SSc to include provision for those that are AA negative, even when they have undergone extended AA profiling. Our AA negative cohort was clinically similar to other published AA negative cohorts in that they have more fibrotic features and are less likely to have the SSc associated vasculopathy characterised by Raynaud's phenomenon, telangiectasias, and to a lesser extent, digital ulcers. Males also featured prominently in this subset. We did not find an association with malignancy but others have commented on a temporal association (343) and it seems prudent to screen any patient presenting atypically for other causes of disease.

Platelet Derived Growth Factor Receptor AA

Platelet derived growth factor receptor (PDGFR) was positive in only two patients in our cohort but deserves mention in this summary as detecting its presence had been an initial aim of this thesis. Both patients who were PDGFR positive had AAs that were of higher staining intensity and were stratified to either Cluster 3 Topo or Cluster 5 CENP. There were no clinical characteristics that were evident in this cohort that could be identified as being unique to PDGFR. The same was found in other cohorts globally regardless of genetic or geographic background (76, 116, 144, 200). The LIA might not be the best type of assay to detect this AA and it may be more useful to replace PDGFR with U1RNP.

Line Blot Technology as a Method of Autoantibody Detection

The Euroimmun line immunoblot assay (LIA) is an established technology that has been validated in a variety of cohorts of various ethnic and genetic backgrounds. It compares favourably with Australian diagnostic laboratory testing. We used a single operator, single platform approach and testing by independent laboratories had excellent agreement for Topo1 (κ=0.837) and CENP (κ=0.887) and good to substantial agreement for RNAP3 (κ = .775). Fewer comparisons for the RNAP3 assay were available because many central and regional laboratories did not have RNAP3 testing routinely available at the time sera were tested for the ASCS. The lower kappa statistic for RNAP3 may also reflect that these AA titres are known to change over time as the sera used for this comparison study was not taken at the same time point.

Where there was discordance among the different testing methods it was most often due to multiple AA staining, with lower intensity staining being discordant and in these instances the highest staining intensity scores were most often concordant with ASIG results. The Immunoblot is more sensitive to AA detection with the added advantage of multiple AA recognition; although the clinical interpretation of multiple AAs requires further consideration. This initial work has shown that the LIA has a role in AA sub-classification and our comparison with other testing platforms can provide reassurance that other testing platforms such as ELISA, IP and IIF provide comparable results, as long as the AA with the highest titre AA is considered.

The use of autoantibodies may provide a more meaningful stratification of scleroderma subsets than the currently used limited, diffuse and overlap classification system, particularly in very early disease. This may have particular relevance when identifying patients for clinical trials or guiding monitoring for those more likely to develop organ specific complications.

Future Directions

Patient stratification systems

In this study it was evident that the presence and staining intensity of AAs corresponded with clinical characteristics at least where the three primary AAs (Topo1, CENP and RNAP3) were concerned. Therefore using AAs as a primary sub classification tool may provide some benefits over the current limited, diffuse and overlap classification system, particular in very early disease before clinical features are apparent. However, it is also evident that the extent of skin fibrosis is important in SSc (26, 283) and variation in the extent of skin involvement reflects varied clinical phenotype. A sub classification system that uses both AA subset and the modified Rodnan Skin Score may be more useful in understanding patient subgroups and

individual patients. In this way, a utilitarian report of the patient is available for further prognostication and also how the patient is characterised for treatment.

Further characterisation of patients with monospecific and multiple autoantibody positivity

Many patients co-express AAs as was evident in this cohort where half the patients had 2 or more AAs. Where numbers permitted, we characterised clinical manifestations of monospecific AA expression. To further characterise the effects of multiple AA expression, a better understanding of monospecific expression is required, and this requires large multinational collaborations. Potentially, patients may be further characterised by both their primary AA and subsequently by their co-expressed AAs.

Titres and staining intensity – high and low, stability and mapping the clinical course

Autoantibody titres are thought to remain stable over the course of disease (246); however there are reports that challenge this finding. Both Topo1 (136) and RNAP3 (157) are reported to fluctuate in individual patients and the change in titre (increase or decrease) was correlated with clinical expression of disease. CENP has been reported to be relatively stable over the disease course (101) but changes in titre in other AAs are unknown. In this study, the AA staining intensity stratified patients to specific clusters. It would be interesting to take serial AA titres or staining intensity measures from patient in different clusters over time and map the clinical course of disease. This would generate an understanding of variability in AA levels and also potentially identify responders and non-responders to treatment if AA levels follow the course of disease.

Improve detection methods and explore new epitopes of SSc AAs such as fibrillarin and Th/To.

Detection of fibrillarin is reported to be challenging as there is an antigenic complexity to the C/D box snoRNPs (204) and there is emerging research that there are other Th/To protein targets (82) that may identify more patients with this AA. At this point, both of these AAs were relatively rare in this Australian cohort and while this could be attributed to genetic and ethnic background, there may be other epitopes that may identify more patients that express this AA. Continuing research to identify new epitopes and assays for diagnostic use is required.

Characterising AAs for ANA only positive patients

There remained 33 ANA positive patients that did not have a detectable AA in Cluster 4. Further characterisation of these patients is required to detect specific AA positivity. Some of these patients may be positive for the new AAs that have been identified in SSc cohorts (see Chapter 1 Table 1-7 pp.51-52). Clinical characterisation and multinational collaborations are required to identify patients that are positive for these newer AAs.

Further characterisation of specific subgroups

Further characterisation of specific subgroups identified in Cluster 4 such as the AA negative group and the double positive Topo1/CENP subgroup will give a better understanding of disease course in these patients.

Cluster Outcomes

The ASIG is a relatively new database (2007) and future studies would include survival and cause of death. This would benefit clinicians in giving advice as to prognostication.

The ageing immune system, cellular senescence and TRIM21/Ro52

Systemic sclerosis is not viewed as a disease of ageing, yet many of the clinical characteristics have features in common with ageing. Features that are typically viewed in relation to age are genomic instability and DNA damage, (359) pulmonary and systemic hypertension, renal impairment, fibrosis, cardio-pulmonary disease and muscle weakness. Older SSc patients, that is patients who present over the age of 65, have an increased risk of mortality from one of these features (360).

Ageing is not a passive degenerative process; it is a complex process involving most physiological systems of the body. The immune system has important physiological and regulatory responses and it is well documented that many aspects of immune responses are decreased or increased with ageing resulting in dysregulation (361).

TRIM21/Ro52 patients experience Raynaud's at an older age and have an older age of disease onset (241) and it is the most commonly co-expressed autoantibody. It has an important role in the ubiquitin process and it has the ability to regulate downstream signalling of various pattern recognition receptors including NF-κβ, TGF-β and interferon response. There may be underlying senescence mechanisms (331, 332) that are yet to be elucidated that are connected with this abundantly expressed AA. It would be interesting to investigate the ageing immune system in SSc in association with the AAs, particularly TRIM21/Ro52.

Personalised Medicine

Scleroderma, as with most autoimmune conditions, has a suite of biomarkers associated with various pathologies within the condition (51). Serum autoantibodies, found in >95% (65) of patients, are correlated with distinct clinical manifestations and have the potential, particularly in early disease, to aid in predicting disease course. There is potential to utilise AAs in conjunction with other biomarkers to predict fibrotic, vascular and organ manifestations and response to treatment. For example a panel of SSc associated AAs could be interpreted in combination with information obtained from transcriptomics, proteomics, metabolomics, genomics and epigenomics to provide a detailed and individually personalised assessment of disease course (13) . Theoretically this will be highly beneficial for patient outcomes as biomarkers based on precision medicine will refine treatments and therapies for which the patient is most likely to respond (66).

Final Comments

The principal aim investigated in this thesis has been to determine the frequency and clinical associations of SSc related autoantibodies in a well-characterised Australian scleroderma cohort using the commercially available Line Immuno- Assay.

It can be concluded that a variety of AAs are found in scleroderma, some of which are linked with discreet clinical phenotypes, and that using the statistical technique of PCA these AAs can be used to stratify patients into five distinct “clusters” with potential clinical utility. Further, some patients were identified with multiple AA positivity and in a small group of patients no antibody was detected. The relevance of both these findings was explored. Co-expression of Topo1/CENP forms a small but unique subgroup and in the Australian cohort antibodies to PDGFR are very rare. Finally, it has been determined that the LIA has good concordance with other commercially available assays and that the addition of U1RNP to the current LIA range of antigens might improve the diagnostic utility of this assay in detecting scleroderma associated antibodies.

Figure 7-1: Paul Klee 1879-1940: Capture c1935

> *Paul Klee was an artist and musician. His work expressed examples of Cubism, Expressionism and Surrealism. At one time he taught at the famous German art and design school, ‘Bauhaus’. From 1935 his work expressed his feelings on the changes he went through, both physically and psychologically, in having what was thought to be, diffuse scleroderma.*

This completes the thesis ‘*The Utility of Autoantibodies as Biomarkers in a well characterised Australian Systemic Sclerosis (scleroderma) cohort’*. While much has been learnt, there is still much to learn.

APPENDICES

Thesis publication

i. Patterson, K.A., Roberts-Thomson, P.J., Lester, S., Tan, J.A., Hakendorf, P., Rischmueller, M., Zochling,J., Sahhar, J., Nash, P., Roddy, J., Hill, C., Nikpour, M., Stevens, W., Proudman, D.M., Walker, J.G. *Interpretation of an Extended Autoantibody Profile in a Well-Characterized Australian Systemic Sclerosis (Scleroderma) Cohort Using Principal Components Analysis.* Arthritis and Rheumatology. 2015; 67(12):3234-44

Contributions to other papers from work in this thesis

i. Carolina Mejia Otero, Shervin Assassi, Marie Hudson, Maureen D. Mayes, Rosa Estrada-Y-Martin, Claudia Pedroza, Tingting W. Mills, Jennifer Walker, Murray Baron, Wendy Stevens, Susanna M. Proudman, Mandana Nikpour, Sonal Mehra, Mianbo Wang, and Marvin J. Fritzler; Canadian Scleroderma Research Group (CSRG); Australian Scleroderma Cohort Study (ASCS); Genetics versus Environment in Scleroderma Outcome Study (GENISOS) *Anti-fibrillarin Antibodies Are Associated with Native-North American Ethnicity and Poorer Survival in Systemic Sclerosis.* Journal of Rheumatology. Accepted 2016. Manuscript number: 2016-0574.R1

ii. Wodkowski M, Hudson M, Proudman S, Walker J, Stevens W, Nikpour M, Canadian Scleroderma Research Group (CSRG); Australian Scleroderma Cohort Study (ASCS); Genetics versus Environment in Scleroderma Outcome Study (GENISOS). *Clinical correlates of monospecific anti-PM75 and anti-PM100 antibodies in a tri-nation cohort of 1574 systemic sclerosis subjects.* Autoimmunity. 2015;48(8):542-51

iii. Wodkowski M, Hudson M, Proudman S, Walker J, Stevens W, Nikpour M, Canadian Scleroderma Research Group (CSRG); Australian Scleroderma Cohort Study (ASCS); Genetics versus Environment in Scleroderma Outcome Study (GENISOS). *Monospecific anti-Ro52/TRIM21 antibodies in a tri-nation cohort of 1574 systemic sclerosis subjects: Evidence of an association with interstitial lung disease and worse survival.* Clinical and Experimental Rheumatology. 2015;33:131-5.

iv. Mehra, S., Walker, J.G., Patterson, K.A., Fritzler, M.J. *Autoantibodies in Systemic Sclerosis* Autoimmunity Reviews. 2013;12(3):340-54.

Contributions to Abstracts from work in this thesis

i. Walker JG[1], Nikpour M[2,3], Huq M[3], Patterson K[1], Roberts-Thomson PJ[1], Proudman S[4,5], Stevens W[6], Lester S[7,8], Rischmueller M[4,8], Zochling J[9], Sahhar J[10,11], Nash P[12], Roddy J[13], Hill C[4,8], Hudson M[14], Baron M[14], Pope J[15], Mayes MD[16], Assassi S[16], Mahler M[17],Fritzler MJ[18]; Canadian Scleroderma Research Group, Genetics versus Environment in Scleroderma Outcome Study, Australian Scleroderma Interest Group. *Autoantibodies to the hPOP1 and Rpp25/38 components of the Th/To complex identify a subgroup of systemic sclerosis (SSc) associated interstitial lung disease (ILD) and antibodies to hPOP1 are associated with reduced survival.* Submitted to American College Rheumatology Conference, San Diego, California, USA, November 2017.

ii. S Lester, K Patterson, J Walker, JC Charlesworth, J Stankovich, W Stevens, J Sahhar, P Nash, J Roddy, C Hill, M Nikpour, M Rischmueller, S Proudman, M Brown, J Zochling. *Associations between HLA DRB1 Alleles and Autoantibodies in Systemic Sclerosis.* Internal Medicine Journal. 2014;44:2-2

Australian Scleroderma Interest Group Terms of Reference

June 2013

The Steering Committee of the Australian Scleroderma Interest Group (ASIG) first met in November 2005, and in 2007 it became a special interest group under the auspices of the Australian Rheumatology Association (ARA), a not-for-profit organisation incorporated as a company limited by guarantee in Australia. In January 2012, ARA-ASIG was granted an ABN and registered with the Australian Business Register as an Other Incorporated Entity (ABN: 54 709 736 928). ARA-ASIG is governed by an Executive Committee that operates according to the Constitution of the ARA.

1. Mission Statement

The ASIG consists of physicians interested in improving the care of patients with scleroderma or systemic sclerosis (SSc). This will be achieved by clinical research and the development of guidelines for the investigation, monitoring and management of patients with SSc in accordance with current best practice. The implementation and effectiveness of these guidelines in improving survival and quality of life of SSc patients in Australia will be monitored and recursively modified according to new evidence as it arises from research activities.

2. Background

Scleroderma and mixed connective tissue disease (MCTD) are rare, chronic disease characterised by autoimmunity, vasculopathy and fibrosis. Approximately one quarter of SSc/MCTD patients may develop very serious complications - including pulmonary arterial hypertension (PAH) and/or interstitial lung Disease (ILD). PAH is a disease of lung blood vessels which become narrowed and reduce blood flow through the lungs. In ILD, the walls of the air sacs in the lung and the lung tissue become inflamed and if this inflammation continues then scarring, otherwise known as fibrosis, occurs and the lungs become stiff.

The typical symptoms of PAH and ILD are shortness of breath, dizziness, fainting, and other symptoms which impact greatly on quality of life and limit daily activities. In the late stages of PAH, the blood vessel changes in the lungs lead to right-sided heart failure.

PAH and ILD are recognised as the number one cause of death in patients with SSc[1]. Reports of the prevalence of SSc and of PAH associated with SSc vary. It has been estimated that in Australia, the point prevalence of SSc is approximately 2300 patients[2] and that SSc PAH in Australia could be expected to affect approximately 600 patients[3], confirmed by a study that found the prevalence of PAH within the SSc or MCTD population to be 26%[4]. Furthermore, there is evidence that PAH is under-diagnosed, with recent studies suggesting more than 13% of patients followed up in a community rheumatology setting had undiagnosed PAH prior to formal assessment[4].

The disease process in the pulmonary arteries which leads to PAH can occur over several years. The signs and symptoms of this disease are often hard to distinguish from other conditions, particularly in the early stages. This frequently delays the formal diagnosis of PAH which is made by right heart catheterisation. As well as the condition often being silent until late in the disease, it may not be detected by less-invasive investigations such as echocardiogram (ECHO) if there is no minor leakiness of the tricuspid valve on the right side of the heart (approximately 25% of people) or if the technicians performing the investigations do not have the specific skills required for detecting PAH.

There is now effective treatment available[5-8] which can reduce the severity of the complications of this disease if provided early in the illness, so timely and ongoing screening for SSc patients is currently recommended by the British Thoracic Society[9], American College of Chest Physicians[10] and the Royal Free Hospital Connective Tissue Disease Clinic.

Prior to the commencement of the ASIG screening programs in 2007, screening was not consistently offered in Australia, according to an agreed protocol.

3. Scope

The ASIG will, as appropriate, undertake projects that assist in meeting the mission of the group. The findings of the projects will be reported to the ARA distributed to patient groups published in relevant peer-reviewed journals

4. Specific issues to be addressed

The ASIG successfully achieved the priority areas identified for the period of 2007 to 2012:

The development of the Australian Scleroderma Screening Program (ASSP).

The ASSP utilised a screening protocol developed by the group, based on international best practice. Screening commenced at designated centres around Australia that were established according to the standards of the protocol and overseen by a member of ASIG. All Australian physicians treating patients with SSc/MCTD have been invited to refer their patients for routine annual screening, with the referring physician retaining responsibility for the ongoing management of their own patients. As the screening is considered part of routine care, this priority area will continue indefinitely; however the ASSP Protocol will be reviewed periodically to ensure that it continues to reflect international best practice.

The implementation of the ASSP Research Project – the Australian Scleroderma Cohort Study (ASCS)

The aim of the research project is to collate de-identified data collected from the screening centres for investigating whether there are predisposing factors or markers which can be used to predict the risk of developing pulmonary complications of SSc. Ethics approval was granted at each participating centre and data collected into an online database from December 2007. Aggregated de-identified data have been analysed and used in national and international presentations.

The research outcomes are being used to develop and review guidelines for providing best care for patients. The analysis of the data will also be used to inform subsequent modifications of the ASSP Protocol.

Ongoing priority areas for the period 2010 to 2012 were as follows:

i) Annual screening of all patients diagnosed with SSc or MCTD remained a priority for the ASIG.

More than 1300 patients had elected to participate in a screening program during by end 2012, Screening is ongoing for most of these patients. Additional centres have joined the collaboration as listed in point 6.0. New centres are welcome to join at any time or physicians may refer patients for screening services at existing centres.

ii) The ASCS

Most of the screened patients have also consented to participate in the ASIG research project, ASCS. An electronic database was established in December 2007 to collect aggregated de-identified data and a blood sample storage arrangement commenced with the Arthritis Research Laboratory in Adelaide in 2008. Patients consented to the research have been enrolled consecutively. While further recruitment into the research project is an option, the collaboration has identified the need to focus on the collection of detailed longitudinal data on existing research participants.

Detailed data collection on existing consented patients is a priority for the research project.

a) The exploration of collaboration with international groups. Collaborations are being explored or have been established with groups in Canada, the US and the UK.
b) Data analysis and publication. Several publications are in preparation, have been submitted and/or have been accepted for publication in peer-reviewed journals. Many more are in the pipeline.
c) Extending the funding sources beyond pharmaceutical companies. Applications for competitive funding continue to be made with project grant applications to NHMRC and Arthritis Australia. Successful applications have also been made to industry.

Ongoing priority areas for the period 2012 to 2013 are as follows:

a) Ensuring participation in screening a large proportion of SSc patients that is representative of the Australian population
b) It has been recognised that not all states are well-represented in the ASCS and that efforts to support the sustainable expansion of existing centres and/or develop new centres is a priority.
c) Blood research priority – collecting samples and storing data
d) Establishment and improvement of blood sample collection and processing at each centre.
e) Development of the ASIG blood biobank

Attracting research funds

- NHMRC
- Sponsorship form pharmaceutical companies
- Donations

Formulating strategies to assist smaller sites in data collection and follow-up

5. Desired outcomes/outputs

The aims of ASIG are:

- Development and ongoing refinement of guidelines for the screening of patients with SSc/MCTD
- Establish the prevalence of PAH in Australian patients
- Improve the outcomes for patients with complications of SSc through early detection
- Contribute to epidemiological research into predisposing factors for serious complications
- Contribute to an understanding of the most effective treatment therapies
- Set up a process for linking data nationally that could be used for future research projects
- Maintain a multidisciplinary collaboration for the benefit of SSc research.

6. Persons involved

The ASIG collaboration consists of the following members:

- A/Prof Susanna Proudman, *Chair Executive Committee* (2007) and Dr Jenny Walker (2009), Royal Adelaide Hospital, SA
- Dr Wendy Stevens, St Vincent's Hospital, VIC – *Secretary* (2007)
- Dr Janet Roddy and Dr Madelynn Chan, Royal Perth Hospital, WA (2007)
- A Prof Catherine Hill, Queen Elizabeth Hospital, SA (2007)
- A/Prof Peter Nash, Sunshine Coast Rheumatology, QLD (2007)
- A/Prof Peter Youssef, Royal Prince Alfred, NSW – *Chair Scientific Committee* (2007), and Dr Tamara Corte, Respiratory Physician (2011)
- A/Prof Allan Sturgess, St George, NSW (2007)
- A/Prof Les Schrieber, Royal North Shore, NSW - *Treasurer* (2008)
- Dr Gabor Major, John Hunter, NSW (2008)
- A/Prof Glenn Reeves, John Hunter, NSW (2008)
- A/Prof Kathleen Tymms and Dr Anna Dorai Raj, Canberra Rheumatology, ACT (2007)
- Dr Joanne Sahhar, Monash Medical Centre, VIC (2007)
- Dr Jane Zochling, Menzie's Institute, TAS (2007)
- Prof David Celermajer, Royal Prince Alfred, NSW – Cardiologist (2007)
- Dr Mandy Nikpour, St Vincent's Hospital – Rheumatologist & Epidemiologist (2009)
- Ms Sue Lester and Dr Maureen Rischmueller, The Queen Elizabeth Hospital, SA – Biobank (year of first involvement 2007)

Previous members of the collaboration who contributed to the development of ASIG:

- Assoc Prof Eli Gabbay, Royal Perth Hospital, WA – Respiratory Physician

Previous members of the collaboration who contributed data:

- Dr Fiona Kermeen, Prince Charles Hospital, QLD (2007 – 2009)

Physicians interested in quality improvement and/or research in SSc are still encouraged to join ASIG, regardless of whether they are members of the ARA or not. Membership of the ASIG Executive Committee is open to physicians who set up a screening centre. If the number of screening centres increases above 15, the number of members of the executive committee will be limited whilst still ensuring equal representation by geographical area. In the case of an issue going to vote, each centre will have one voting right.

All physicians with an interest in SSc/MCTD (mostly but not limited to rheumatologists or immunologists) will be eligible to set up ASSP screening centres, providing they agree to the Terms of Reference, ASSP Protocol, and Research Protocol; and have the collaboration of a cardiologist and respiratory physician.

Members of ASIG provide their time voluntarily to the group.

7. Project Administration

ASIG Executive Committee

The ASIG is managed by an Executive Committee (EC) consisting of the Chair, Treasurer, Secretary (office bearers) and one member from each of the participating centres. Decisions of the EC will be made according to a vote at scheduled meetings or by email between meetings. A quorum is defined as at least two office bearers and at least two members (total of five). The roles of the EC are to

- Set the strategic direction of ASIG and to provide Governance by developing and monitoring prioriy areas.
- Identify strategies for achieving prioriy areas.
- Administer the group's finances, ensuring a quarterly report is submitted to the ARA.
- Identify funding opportunities.
- Ensure that ASIG operates within the constitution of the ARA.
- Oversee the Project Officer.

The EC receives operational and administrative support from the Project Officer (PO) who reports to the EC at each meeting. The PO has regular meetings with the ASIG office bearers. The PO also provides support to members at each of the ASIG centres in achieving the aims. The ASIG EC meets by teleconference every two to three months throughout the year as required. Teleconferences are generally held in the evening, with members using a dial-in facility charged to the group. Some decisions can be made via email between meetings providing a consensus is reached.

A face to face meeting occurs once a year in conjunction with the ARA Annual Scientific Meeting.

8. ASIG Committees

Scientific Committee

In 2008 a Scientific Committee was established to:

- Devise research strategies for ASIG using ASSP data and patient samples
- Review proposals from other members of ASIG and provide approval before the projects can commence
- Assist with applications for funding to continue the research activities long term

The committee's main goals are:

- To publish research findings related to scleroderma and mixed connective tissue disease
- To mentor other early researchers in this field
- To engage and supervise PhD students or Fellows in the Scleroderma field.

A quorum is defined as at least four members.

Database Committee

In 2009, a Database Committee was established to:

- Oversee the maintenance of the existing ASIG clinical database
- To consider improvements to the clinical database A quorum is defined as at least three members.

Finance Team

In 2012 a Finance Team was established to:

- Approve payments within the ASIG budget

The members of the Finance team have access to on-line banking through CommBiz and are the chair, Susanna Proudman, the treasurer Les Schrieber, Wendy Stevens and Jo Sahhar.

An annual budget is prepared by the PO and approved by the EC at the face to face meeting at the ARA Annual Scientific Meeting in May. Approved budget items will be processed through on-line payment or by credit card with approval from two members of the Finance team. Unbudgeted items of more than $5 000 will need to be approved by the Executive before arrangements are made for them to go ahead.

9. Resources

Funding for the ASIG's primary operations for 2015 and 2016 will be covered by an unrestricted educational grant from Actelion Australia.

Other sources of funding will continue to be explored to enable expansion of the priority areas and to continue the work beyond 2015.

10. Intellectual property and ownership of data

The screening protocol and all instruments contained within it are the property of ASIG (unless otherwise referenced) and cannot be used for purposes other than those associated with the ASSP and ASCS, without written permission from the ASIG executive. ASIG requests that publications arising from the ASIG database reflect the aims of the group and therefore:

- Acknowledge the ASIG collaboration – the group and/or individuals who have contributed to the establishment and ongoing maintenance of the research project, including where relevant, the Biobank.
- Be of a standard that contributes to the successful outcomes of the group to ensure future funding of ASIG for the ongoing maintenance of the database.

In addition, ASIG has developed authorship guidelines (see separate document).

Data from individual centres

Centres are encouraged to analyse data from their own patients obtained from the ASSP database for publication purposes. The ASIG request that in such publications, references to ASIG follow these guidelines.

Posters and oral presentations

There is no need to seek permission for data used in the submission of posters and oral presentations however it is expected that acknowledgement be made that the data were collected from the ASIG database as well as a copy of the abstract sent to the project officer on submission and acceptance.

Manuscripts

With the intention of submission for publication, the ASIG scientific committee would like to be given the opportunity to see the manuscript before submission as any work arising from the ASSP (or a component of it) directly or indirectly represents ASIG on an international level. In reviewing the manuscript, ASIG would like the opportunity to provide constructive comment to the authors for their consideration. The final version should be sent to the committee

prior to submission. ASIG would expect to be acknowledged but has the option to request no acknowledgement if the manuscript is deemed to not meet the standards set by the scientific committee.

In all instances the ASIG committee encourage authors to contact the committee regarding their publication if they would like input or comment from the multidisciplinary experts available. Authorship, including the inclusion of members of ASIG, should comply with current guidelines eg…The choice of authors should be determined early in the planning of the project, or at least early in the preparation of the manuscript.

Aggregated data from all centres

Projects requiring analysis of patient data from other centres obtained from the database require permission from the ASIG scientific committee following a submission of a written research proposal on the relevant ASIG form.

Posters and oral presentations

All posters and oral presentations should list a representative from each centre and acknowledge "*Australian Scleroderma Interest Group (ASIG), St Vincent's Hospital IT Dept an unrestricted educational grants from Actelion Australia, CSL Biotherapies, Bayer, Pfizer."* (to be modified to include any donors of funds used for the study in question). Where there is a limit on number of authors, the centres should be rotated. A copy of the abstract should be sent to the project officer on submission who should then be informed regarding its acceptance or not.

Manuscripts

With the intention of submission for publication, ASIG requests that manuscripts be sent to the project officer for perusal by the scientific committee prior to submission as any work arising from the ASSP (or a component of it) directly or indirectly represents ASIG on an international level. In reviewing manuscripts with aggregated data, the scientific committee would like the opportunity to contribute constructively and expects to advise regarding appropriate selection of ASIG executive members for authorship, based on a significant contribution to the development phase of ASSP or direct involvement in the study being submitted for publication. Should the manuscript be deemed to not meet the standards set by the scientific committee it will be recommended that it not be submitted for publication.

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Rules for authorship of the Australian Scleroderma Interest Group

This authorship policy pertains to all publications or abstracts produced using data from more than one ASIG site obtained from the ASIG central database or using sera or biologic specimens from either the central ASIG bio-bank or from more than one ASIG site. This is an official document of the ASIG and was approved/amended on the date at the bottom.

The screening protocol and all instruments contained within it are the property of ASIG (unless otherwise referenced) and cannot be used for purposes other than those associated with the ASSP and ASCS, without written permission from the ASIG executive. ASIG requests that publications arising from the ASIG database reflect the aims of the group and therefore:

- Acknowledge the ASIG collaboration – the group and/or individuals who have contributed to the establishment and ongoing maintenance of the research project.
- Be of a standard that contributes to the successful outcomes of the group to ensure future funding of ASIG for the ongoing maintenance of the database.
- Acknowledge the contribution of members of ASIG who have contributed patients to the publication by listing them as authors whenever possible within the guidelines of the journal.

Therefore ASIG has developed the following guidelines for authorship. A written research proposal for projects using patient data from multiple ASIG sites must be submitted to the Scientific Committee for approval before they can proceed (see request for data and data transfer agreement forms).

These guidelines do not replace the usual processes for determining authorship based on a substantial contribution to a publication. Such a contribution includes listing as authors, individuals responsible for processing and handling of patient samples for publications based on analysis of these samples. It is the responsibility of the lead author to ensure all individuals making such a substantial contribution are included as authors.

Publications or abstracts written by a recruiting rheumatologist using only his/her own data.

The policy outlined below does not apply to publications or abstracts written by a recruiting rheumatologist using only his/her own data extracted from the central database. In that case, the investigator will determine authorship policy and whether the ASIG Chair or nominated senior member of ASIG will be mentioned as an author to recognize the fact that the existence of the database, data quality control and data cleaning procedures are under his/her supervision and these affect the quality of the data

used by the individual investigator. The position of the ASIG director or nominated senior member of ASIG in the authorship list will be at the discretion of the principal author in this situation. If not included in the authorship, it is expected that acknowledgement be made that the data were collected from the ASIG database.

For posters and oral presentations, a copy of the abstract should be sent to the project officer on submission and when known, advice be forwarded concerning acceptance or not.

For manuscripts, the manuscript should be sent to the ASIG Scientific Committee for constructive comment for the authors' consideration. The final version should be sent to the committee prior to submission and when known, advice be forwarded concerning acceptance or not.

ASIG has the option to request no acknowledgement if the manuscript is deemed to not meet the standards set by the Scientific Committee.

General authorship policy for publications using multi-site data within the ASIG.

The primary investigator for the study shall be considered to be the lead author and will be responsible for determining which authors are to be listed and the order of all other named authors except for those mentioned below.

The first and second (or more) author(s) shall be the investigator(s) who have performed most of the work on the particular publication. These names will be determined by the lead author

The Chair of ASIG or nominated senior member of ASIG is a co-author on all manuscripts with his/her name placement a matter of discussion and negotiation with the lead author PRIOR TO submission of the manuscript for publication, unless the Chair of ASIG is the senior or first author

Other named authors will include any person(s) who has contributed substantially to the publication and these names will be determined by the lead author of the study.

If the journal permits, all members of ASIG who have contributed patients to the publication should be listed individually as authors in the same order as described in section C.4. If the journal does not permit every contributing member to be listed as an author, those members who have contributed patients as described in C.1-3. will be listed as authors in the same order as described in section C.4. If the journal does not permit all these authors to be listed, the name "Australian Scleroderma Interest Group" will be included on the authorship line, just preceding the senior author. An indicator such as an *

will be placed next to the group name and, if the journal permits, the names of the members of the group will be listed on the front page of the publication. If the journal does not permit, they will be listed at the end of the publication. If the group name is listed, the corresponding author will make every effort, the publication so permitting, to ensure that all group members will be listed on the NLM pubmed database permitting that name to be searched and associated with this publication on pubmed.

For posters and oral presentations, all members of ASIG who have contributed patients to the publication should be listed individually as authors in the same order as described in section C.4. If there is a limit on the number of authors, the centres should be rotated. A copy of the abstract should be sent to the project officer on submission and when known, advice be forwarded concerning acceptance or not.

Manuscript should be sent to the ASIG Scientific Committee and to all the listed authors for constructive comment for the authors' consideration. The final version should be sent to the committee prior to submission and when known, advice be forwarded concerning acceptance or not.

Advice concerning acknowledgement of financial support (both industry and competitive grant funding) should be sought from the Scientific Committee.

Names to be included under the rubric of the ASIG

The specific names to be included under the rubric of the "Australian Scleroderma Interest Group" will be according to the following rules:

- The author must be a recruiting rheumatologist according to the by-laws of ASIG.
- The author must have contributed complete patient data for a minimum of 5 patients whose data are used in the study.
- The date of visit of the last patient for whom there is complete data in the database entered by an author must be within one (1) year of first submission of the article for consideration for publication. If a recriting rheumatologist has stopped submitting data, then one year after the last data entered he/she will no longer be listed as an author, however, his/her patient information in the database will continue to be used for studies.
- The order of the author list of recruiting ASIG rheumatologists shall be according to the total number of patients recruited with the highest recruiter being named first and the lowest named last. The list will be revised no less frequently than once every 6 months
- Authorship policy for publications for which data has been obtained from other databases as well.

These rules are meant to apply to situations in which ASIG has collaborated with one or more other research groups and the study has involved merging data from more than one source, one of which is ASIG data. The principal investigator(s) of the project and the Directors of each group that contributed data, including the ASIG, must agree on the order of authorship before the ASIG will agree to provide data for the project. The first and second (or more) authors shall be the investigators who have performed most of the work on the particular publication. The primary investigator for the study shall be considered to be the lead author for the study and will be responsible for signing an agreement with the ASIG director determining which other authors are to be listed, and the order of all other named authors. The senior author, who will be listed as the last author, will be at the discretion of the principal investigator. The Chair of ASIG or nominated senior member of ASIG, unless he/she has been listed as first or second author in the list of authors, or he/she has made other specific arrangements with the principal investigator for the study, shall be listed as an author. If other group directors are to be similarly listed, then the order of group directors shall be such that the group that contributed the data from the largest number of subjects will be listed first, the next most data second etc. Other named authors will include any person(s) who has contributed substantially to the publication. These names will be determined by the principal investigator of the study.

In addition, the name “Australian Scleroderma Interest Group” will be included on the authorship line. If other group names are also to be included, then the order of group names shall be such that the group that contributed the data from the largest numbers of patients will be listed first, the next most data second etc…

An indicator such as an * will be placed next to the group name and, if the journal permits, the names of the members of the group will be listed on the front page of the publication. If the journal does not permit they will be listed at the end of the publication. If the journal permits, instead of listing “Australian Scleroderma Interest Group” as an author all names to be included under the rubric of the “Australian Scleroderma Interest Group” may be listed individually as authors in the same order as described in section C. If the group name is listed, the submitter of the publication will make every effort, the publication so permitting, to ensure that all group members will be listed on the NLM pubmed database permitting that name to be searched and associated with this publication on pubmed.

A copy of the abstract should be sent to the project officer on submission and when known, advice be forwarded concerning acceptance or not.

For posters and oral presentations, a copy of the abstract should be sent to the project

officer on submission and when known, advice be forwarded concerning acceptance or not.

Manuscripts should be sent to the ASIG Scientific Committee for constructive comment for the authors' consideration. The final version should be sent to the committee prior to submission and when known, advice be forwarded concerning acceptance or not.

Advice concerning acknowledgement of financial support (both industry and competitive grant funding) should be sought from the Scientific Committee.

Date: June 2013

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