Systemic scleroderma in Greece: low mortality and strong linkage with HLA-DRB1*1104 allele

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Abstract

Objective—Description of Greek patients with scleroderma with reference to (a) major organ disease, (b) autoantibodies, (c) survival rate, and (d) HLA associations.

Methods—The clinical files of 254 patients were analysed retrospectively and a standardised clinical chart was completed with age at disease onset, sex, date of first and last visit, clinical and serological findings, organs affected, reasons for death, and HLA class II alleles. HLA class II alleles (DRB1, DQA1, DQB1, DPB1) were determined by polymerase chain reaction amplification using oligopeptide probes. DNA was extracted from 98 patients and 130 Greek controls.

Results—124 patients (49%) had limited systemic sclerosis (ISSc), 114 (45%) had diffuse systemic sclerosis (dSSc), and 16 (6%) had overlap syndromes. Patients with dSSc, compared with ISSc, were characterised by a higher prevalence of lung disease ($p=0.0011$), oesophageal, heart, and peripheral vessel disease ($p=0.027$, $p=0.0025$, and $p=0.012$, respectively). Anticentromere antibodies (ACA) occurred exclusively in ISSc (34%), whereas antibodies to topoisomerase I (anti-topo I) were associated with dSSc ($p<0.0001$). Anti-topo I were associated with interstitial pulmonary fibrosis, oesophageal and peripheral vessel disease ($p=0.028$, $p=0.012$, and $p=0.01$, respectively). The HLA-DRB1*1104 allele was associated with the disease ($p<0.0001$) and anti-topo I ($p<0.001$), whereas it was not associated with ACA serum reactivity ($p<0.001$). Renal disease occurred in 4% of patients with SSc. The estimated survival probability for this cohort of patients with SSc, four years after the first visit, is 94.8%. **Conclusion**—SSc among Greek subjects has the same pattern of organ disease as in other white populations. However, the prevalence of kidney disease is low. The HLA class II DRB1*1104 allele is associated with the disease, with anti-topo I, and not associated with ACA serum reactivity.

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sclerosis (ISSc) characterised by limited skin disease (hands, face, feet, and forearms), Raynaud's disease occurring many years before the onset of skin changes, mild visceral disease, and a high prevalence of anticentromere antibodies (ACA); and the diffuse systemic sclerosis (dSSc) characterised by truncal skin disease, high prevalence of antibodies to topoisomerase I (anti-topo I), and a more aggressive and rapidly advancing disease course.^{4,5} Furthermore, recently recognised autoantibody specificities, such as autoantibodies to U3 nuclear ribonucleoprotein (fibrillarin)-U3RNP,^{6,7} have been associated with black subjects, skeletal muscle disease, and primary pulmonary arterial hypertension.^{8,9} These factors contribute to the categorisation of patients with scleroderma into subgroups.

Many retrospective studies on survival among patients with SSc have shown that the visceral disease in SSc portends early mortality.^{10–13} A prospective study has shown that after 5.2 years of follow up 50% of patients were dead.¹⁴ However, in a recent report from the United Kingdom the mortality of patients with SSc was not as high as expected on the basis of previous reports.¹⁵ Racial differences and study design are two important reasons for such discrepancies. Black subjects from South Africa are characterised by a complete absence of ACA and they have the severe form of the disease, whereas ISSc is more common in Indians living in South Africa.¹⁶ ACA were shown to be common in white patients with SSc, whereas anti-topo I were common in black patients with SSc.¹⁷ In a report from India describing 78 patients with SSc followed up over 14 years a mortality rate of nearly 6.8% was reported.¹⁸

It has been suggested that anti-topo I or the HLA-DR3/DRw52 haplotype may predict pulmonary fibrosis in patients with SSc¹⁹; the C4A null phenotype provided the strongest association with the disease from the factors of the HLA region²⁰; in addition, the DQA2 allele, which is in linkage disequilibrium with both DR3 and DR11, was also strongly associated with the disease.²⁰

In the light of previous reports this study was undertaken for the following reasons: (a) to describe the prevalence of severe organ disease and the prevalence of various autoantibodies within the Greek SSc population; (b) to associate the clinical and serological findings; (c) to examine the survival rate among Greek patients with scleroderma; (d) to determine whether HLA class II antigens are associated with the disease.

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Systemic sclerosis (SSc) is a multisystemic disorder of unknown cause characterised by progressive fibrosis of the skin, blood vessels, and visceral organs such as the kidneys, lungs, heart, gastrointestinal, and musculoskeletal systems.^{1–3} Two main groups of SSc are recognised—the limited cutaneous systemic

Materials and methods

GENERAL RULES FOR PATIENT FOLLOW UP IN THE OUTPATIENT CLINIC

The referral system

Patients arriving at the outpatient clinics, depending on their symptoms, have either decided to come on their own, or been referred by general practitioners. When a patient has an autoimmune connective tissue disease, or stigmata, he (she) continues to be followed up in our outpatient clinic. The patients whose clinical, serological, and immunogenetic characteristics are presented in this report are consecutive patients followed up in two academic centres of Greece—the department of internal medicine, University of Ioannina and the department of pathophysiology, University of Athens, from 1982 to 1996 and from 1993 to 1996, respectively.

A patient file, which includes clinical and laboratory characteristics, is opened at the first visit; additional information is recorded at each follow up visit. Blood is withdrawn and tested for antinuclear antibodies by immunofluorescence (Hep-2 cells as substrate) and anti-Ro(SSA), anti-La(SSB), anti-Sm, anti-U1RNP, and anti-topo I by counterimmunoelectrophoresis, and Western blotting²¹ using nuclear extracts as previously described.^{22–23} As antigen source rabbit thymus extract was used for the detection of anti-topo I, and calf thymus extract was used for the detection of autoantibodies against other nuclear or cytoplasmic constituents. ACA were detected by indirect immunofluorescence. New antibody systems recently described in SSc, such as anti-U3RNP (fibrillar), and anti-RNA polymerase antibodies were evaluated at a later stage in sera withdrawn from our serum bank. Our anti-RNA polymerase assay was not reproducible and the respective results are not presented in the paper. The results on anti-U3RNP are not presented either because greater numbers of serum samples were available from recent and serious cases than from the older and milder cases, and selection bias could not be avoided.

The evaluation of patients at the first visit includes also a chest x ray, spirometric assessment, and diffusion lung capacity measurements. When there is bilateral reticular pattern of linear or reticulonodular densities in basilar portions of the lungs on chest x ray and/or a restrictive pattern in pulmonary function tests and/or decreased diffusion lung capacity, a high resolution computed tomographic (CT) scan of the chest is performed to confirm the interstitial fibrosis. The National Health care system covers the expenses of the above tests and patient compliance is nearly 85%. At the end of the first visit a provisional classification of the patient's disease as lSSc, dSSc, or overlap syndrome is made. The patients, even in the absence of new complaints, are examined twice a year and have pulmonary function tests at least once a year. Patients with additional, or increasingly severe, complaints are followed up with a complete evaluation every three months or even more frequently.

Clinical charts

A standardised clinical chart was retrospectively completed by taking information from the recorded data in the files of the patients during the entire follow up. Age at disease onset was considered to be the age at which the first signs and symptoms compatible with the disease appeared; such findings or symptoms were Raynaud's disease, puffy hands, sclerodactyly, arthralgias, arthritis, dyspnoea on exertion, and truncal scleroderma.

The criteria for organ disease were based on those previously described.^{12–24} In particular, *peripheral vascular disease* was defined as (a) Raynaud's phenomenon and (b) digital tip ischaemia associated with digital pitting scars, ulcerations or gangrene, or both.

Articular disease was defined as inflammation or tenosynovitis, or both, in more than two joints.

Oesophageal disease was defined as distal oesophageal motility disturbance, suggested by two or more of the following: dysphagia, odynophagia, intermittent heart burn, gastro-oesophageal reflux and/or oesophageal manometric or radiographic abnormalities indicative of oesophageal hypomotility.

Pulmonary disease was established when pulmonary interstitial fibrosis or isolated pulmonary hypertension were found.

Pulmonary interstitial fibrosis was defined on the basis of the following criteria: (a) exclusion of other known causes of alveolar wall fibrosis and (b) bilateral fibrosis confirmed by chest radiograph and/or by high resolution CT scan and/or by restrictive pulmonary abnormalities on pulmonary function tests.

Isolated pulmonary hypertension was defined as clinical evidence of right-heart failure or increased mean pulmonary arterial pressure (>25 mm Hg), or both, recorded by echocardiogram or catheterisation in the absence of severe pulmonary fibrosis or severe myocardial dysfunction.

Isolated impairment of carbon monoxide transfer factor (TLCO) was defined as TLCO less than 70% of the predictive values with the forced vital capacity (FVC) >80% and the FEV₁/FVC ratio >70% of the predictive values (where FEV₁ is the forced expiratory volume in one second), respectively.

Cardiac disease was defined as one of the following: conduction disturbances and/or nodal or ventricular arrhythmias, congestive heart failure not attributable to any other condition and/or moderate to severe pericardial effusion on echocardiogram.

Skeletal muscle disease was defined as proximal muscle weakness recorded on clinical evaluation and any one of the following: raised serum muscle enzyme levels, abnormal findings on electromyography compatible with myositis, histological findings of inflammatory myopathy.

Renal disease was defined as chronic development of renal insufficiency (creatinine clearance less than 45 ml/min on two occasions) or renal crisis defined by a rise in diastolic blood pressure above 110 mm Hg during the final week of observation, associated with

haematuria, or proteinuria, retinal haemorrhages or microangiopathic haemolytic anaemia, or papillo-oedema; antinuclear antibodies, ACA, antibodies to Sm, U1RNP, Ro(SSA), La(SSB), and anti-topo I, C3 and C4 complement levels, cryoglobulins, rheumatoid factors; and the HLA-DRB1, DQA1, DQB1, and DPB1 alleles of the patients.

Finally, the outcome expressed in terms of death, renal, cardiac, and pulmonary failure was recorded.

Patient classification

The skin disease was evaluated by two of us (PGV and IP) who were running the outpatient clinic in Ioannina (PGV) and Athens (PGV and IP). The categorisation of patients as dSSc and lSSc was based on their latest evaluation in the clinic. According to the extent of skin disease and additional findings characterising the systemic form of the disease, the patients were classified as those with lSSc (sclerosis distal to elbows and knees and/or face) or as those with dSSc (truncal skin disease). Patients with features of myositis or systemic lupus erythematosus related features, or both, were classified as having the scleroderma overlap syndrome.

CLASS II DRB1, DQA1, DQB1, DPB1 TYPING

The patients were contacted by phone for blood collection for DNA extraction. DNA was collected from the 98 patients who responded between 1 January and 31 December 1995. To ensure that the HLA typed patients comprised a representative group of our cohort, the major disease characteristics of these patients were compared with those of the patients not typed for HLA. It was found that the two groups were comparable for disease subgroup ($p=0.095$), sex ($p=0.079$), pulmonary disease ($p=0.269$), renal disease ($p=0.426$), heart disease ($p=1.0$), severe peripheral ischaemia, responsible for digital ulcers or gangrene ($p=0.288$), anti-topo I ($p=0.083$), anti-U1RNP ($p=0.215$), and ACA positivity ($p=0.486$). The only difference was in the incidence of oesophageal disease ($p=0.003$).

Cell lines

DNA from HLA-D homozygous B lymphoblastoid cell lines, fully defined during the XIIth International Histocompatibility Workshop, were used as reference.²⁵

DNA extraction

DNA was prepared from anticoagulated venous blood by a salting out method.²⁶

Amplification of HLA class II alleles

DNA amplification was carried out by the polymerase chain reaction (PCR) method according to Saiki *et al.*²⁷ The PCR reaction mixture consisted of 1 µg of genomic DNAs, PCR buffer (100 mM Tris pH 8.5, 500 mM KCl, 20 mM MgCl₂, 0.1% gelatin), 0.2 mmol/l of each dNTP, 1 ng of each primer, which had been designed to amplify the second exon of DRB1, DQA1, and DQB1 genes (primers and probes were defined by the XIIth International Histocompatibility Workshop²⁵), and 2.5 units of Taq DNA polymerase (Perkin-Elmer/

Cetus). The mixture was covered with an equal volume of liquid paraffin and then submitted to 30 cycles using the Perkin-Elmer/Cetus thermocycler model. Specifically, the following primer pairs were used, according to the XIIth International Histocompatibility Workshop: for the DRB generic 2DRBAMP-A and 2DRBAMP-B; for DR4 subtyping 2DRBAMP-4 and 2DRBAMP-B; for DR2 subtyping 2DRBAMP-2 and 2DRBAMP-B; for DR1 subtyping 2DRBAMP-1 and 2DRBAMP-B; for DR52 subtyping 2DRBAMP-3 and 2DRBAMP-B; for the DQA1 gene 2DQAAMP-A and 2DQAAMP-B; for the DQB1 gene 2DQBAMP-A and 2DQBAMP-B; and for DPB generic 2DPBAMP-A and 2DPBAMP-B.

Determination of amplified DNA

Amplified DNA samples were denatured and dot blotted onto positively charged nylon membranes. The respective membranes were hybridised with sequence-specific oligonucleotide probes (SSO) as indicated, allowing relatively high resolution of the known alleles. SSO probes were 3' end labelled with digoxigenin-dideoxyuridine triphosphate and DNA deoxynucleotide transferase. The membranes were hybridised for one hour at 54°C in 3 M tetramethylammonium chloride (TMACl) solution (5 × SSPE, 5 × Denhardt's, 0.1% sodium dodecyl sulphate (SDS), and 3 M TMACl) with the end labelled SSO probe and then washed in 3 M TMACl buffer (50 mM Tris.HCl pH 8.2 mM (EDTA), 0.1 % SDS, 3 M TMACl) for 15 minutes at either 58°C (18-mers) or 59°C (19-mers), or 60°C (20-mers). Membranes were incubated with a sheep Fab anti-digoxigenin IgG fragment conjugated to alkaline phosphatase and the detection was done using substrate producing chemiluminescence after enzymatic reaction with 3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD). Dots were visualised after exposure for 15 minutes to two hours to Kodak x ray films at room temperature, according to the appearance of positive and negative controls.²⁸

STATISTICAL ANALYSIS

Kruskal-Wallis and Fisher's exact tests were used for comparisons of the continuous and discrete measures, respectively, between different SSc subgroups. Time to event data were compared using the log rank test, and product-limit estimates were calculated by the Kaplan-Meier method. Log linear models were used to explore the association of different auto-antibodies to major organ disease and to SSc subgroups. Reported p values are two sided. Bonferroni adjusted significance levels for comparisons between allelic frequencies in lSSc and dSSc groups are reported. The SAS and StatXact statistical packages were used in the analysis.^{29 30}

Table 1 Characteristics of Greek patients with scleroderma by scleroderma type

Characteristics	ISSc* (n=124)	dSSc* (n=114)	Overlap (n=16)
Female† (No (%))	114 (91.9)	95 (83.3)	12 (75.0)
Age at onset (years)			
Mean (SE)	41.8 (1.3)	38.4 (1.4)	45.3 (4.6)
Median	44	38	48
Min-max	10–81	10–72	8–66
Disease duration (years)			
Mean (SE)	11.5 (0.9)	9.1 (0.8)	9.3 (1.9)
Median	9.0	6.0	7.5
Min-max	<1–97	<1–50	<1–35
Duration of follow up (years)			
Mean (SE)	2.5 (0.34)	3.0 (0.34)	3.0 (0.54)
Median	1.0	2.0	2.0
Min-max	<1–14	<1–13	<1–8

*ISSc = limited systemic sclerosis; dSSc = diffuse systemic sclerosis.

†p=0.049 by Fisher's exact test for comparison between dSSc and ISSc.

Results

DEMOGRAPHIC FEATURES AND DIAGNOSIS OF PATIENTS WITH SCLERODERMA

Between 1982 and 1996 254 patients with scleroderma (221 (87%) female, 33 (13%) male; F:M 6.7:1) were identified in two academic medical centres of Greece. Of these patients, 124 patients (49%) had ISSc, 114 (45%) had dSSc, and 16 (6%) had overlap syndrome. The mean age at disease onset for these groups was 41.8, 38.4, and 45.3 years and mean duration of the disease 11.5, 9.1, and 9.3 years respectively. The corresponding mean follow up period is 2.5, 3.0, and 3.0 years respectively. Table 1 summarises the characteristics of the patients according to disease subcategory. A higher percentage of male patients were diagnosed as having dSSc rather than ISSc. Duration of the disease was calculated from the date of the first symptom, whereas disease follow up was from the first visit. Age at disease onset, disease duration, and follow up duration did not significantly differ in the two major disease subgroups ($p=0.07$, $p=0.09$, and $p=0.51$, respectively). In our cohort, scleroderma occurred in the third to fifth decade of life for 60% of the patients.

MAJOR ORGAN DISEASE

The peripheral vessels were affected in patients with scleroderma more often than any other system. In particular Raynaud's phenomenon occurred in almost all the patients. The prevalence of severe digital ischaemia, expressed as

Table 2 Major organ disease in Greek patients with limited (ISSc) and diffuse (dSSc) systemic sclerosis. Results are shown as No (%)

Organ affected	ISSc (n = 124)	dSSc (n = 114)	Fisher's exact p value
Raynaud's phenomenon	123 (99)	113 (99)	NS
Lung†	32 (32)	58 (56)	0.0011
Kidney	3 (2)	7 (6)	NS
Oesophagus	50 (40)	62 (54)	0.027
Heart	9 (7)	24 (21)	0.0025
Digital pitting scars/ulcers/gangrene	63 (51)	77 (68)	0.012
Joints	32 (26)	34 (30)	NS
Peripheral neuropathy	2 (2)	3 (3)	NS
<i>Pulmonary evaluation</i>	(n = 99)	(n = 104)	
Interstitial fibrosis	28 (28)	57 (55)	0.0003
Isolated pulmonary hypertension	4 (4)	1 (1)	NS
Isolated reduction of TLCO*	7 (7)	12 (12)	NS

*TLCO = carbon monoxide transfer factor.

†For patients with pulmonary evaluation n = 99 for ISSc and 104 for dSSc.

digital pitting scars, digital ulcers, or gangrene, oesophageal disease, and pulmonary disease was significantly higher in dSSc than in ISSc (table 2). When the patterns of pulmonary disease were evaluated, it was shown that interstitial fibrosis more commonly affected the dSSc and overlap subgroups than the ISSc subgroup ($p=0.0003$, table 2). Isolated pulmonary hypertension was evident in 4% of patients with ISSc, in 1% of the patients with dSSc, and was not found in patients of the overlap subgroup. The prevalence of joint disease did not differ in dSSc and ISSc subgroups. Peripheral neuropathy occurred in a minority of our patients with no differences in prevalence among the scleroderma subgroups. The heart was affected in over one fifth of patients with dSSc; this prevalence was significantly higher than that observed in patients with ISSc ($p=0.0025$, table 2). Renal disease occurred in 7/114 (6%) patients with dSSc and 3/124 (2%) patients with ISSc—a total of 10/234 (4%) patients with SSc. Renal crisis occurred in 3/124 (2%) patients with dSSc—1% of the total number of patients with SSc. These three patients had severe and extensive scleroderma and were also positive for anti-topo I.

A number of patients who were completely evaluated for pulmonary disease had a severe reduction of TLCO as stated in "Materials and methods", but no evidence of isolated pulmonary hypertension or pulmonary fibrosis. In fact 7/99 (7%) patients with ISSc and 12/104 (12%) patients with dSSc expressed isolated reduction of TLCO (table 2). The incidence, after the first visit, of oesophageal disease, cardiac disease, and pulmonary disease is estimated as 25.8, 4.6, and 14.3 per 100 patient-years of follow up, respectively.

AUTOANTIBODIES IN THE SERA OF GREEK PATIENTS WITH SSC

It was shown that ACA occur exclusively in patients with ISSc; a total of 42/124 (34%) patients with ISSc were positive for ACA but none of the dSSc or overlap patients ($p<0.0001$). Anti-topo I occurred in 68/114 (60%) of patients with dSSc and also in 26/124 (21%) of patients with ISSc; finally, 6/16 (38%) patients with overlap syndrome were positive for anti-topo I. Antibodies to U1RNP occurred in 4/16 (25%) of overlap patients with scleroderma compared with 10/124 (8%) and 5/114 (4%) of patients with ISSc and dSSc, respectively ($p=0.019$). In addition, anti-Ro(SSA) occurred more commonly in overlap patients with scleroderma (4/16

Table 3 Autoantibodies in the Greek patients with scleroderma. Results are shown as No (%) of patients

Autoantibody	ISSc* (n=124)	dSSc* (n=114)	Overlap (n=16)	Fisher's exact p value
ACA*	42 (34)	0 (0)	0 (0)	† <0.0001
Anti-topo I	26 (21)	68 (60)	6 (38)	† <0.0001
Anti-Ro(SSA)	13 (10)	7 (6)	4 (25)	‡ 0.043
Anti-La(SSB)	6 (5)	2 (2)	2 (13)	‡ 0.073
Anti-U1RNP	10 (8)	5 (4)	4 (25)	‡ 0.019

*ISSc = limited systemic sclerosis; dSSc = diffuse systemic sclerosis; ACA = anticentromere antibodies.

† Comparisons between ISSc and dSSc.

‡ Comparisons between overlap and dSSc.

Table 4 HLA-DR and DQ antigens in Greek patients with scleroderma (SSc) and controls. Results are shown as No (%) of alleles

HLA alleles corresponding to:	SSc (n=98) Alleles (n=196)	Controls (n=130) Alleles (n=260)	Odds ratio	Fisher's p value
DR*				
DR1	11 (6)	24 (9)	1.710	0.16
DR2	40 (20)	62 (24)	1.221	0.43
-DR15	17 (9)	18 (7)	0.783	0.48
-DR16	23 (12)	44 (17)	1.532	0.14
DR3	13 (7)	16 (6)	0.923	0.85
DR4	19 (10)	25 (10)	0.991	1.00
DR5	82 (42)	65 (25)	0.463	0.0002
-DR11	81 (41)	65 (25)	0.463	0.0003
-DR12	1 (0.5)	0 (0)	—	0.43
DR6	11 (6)	32 (12)	2.360	0.015
-DR13	7 (4)	22 (8)	2.426	0.050
-DR14	4 (2)	10 (4)	1.920	0.41
DR7	9 (5)	19 (7)	1.638	0.25
DR8	7 (4)	5 (2)	0.524	0.38
DR9	—	—	—	—
DR10	4 (2)	6 (2)	1.134	1.00
DQ†				
DQ2	23 (12)	32 (12)	1.056	0.89
DQ4	5 (3)	3 (1)	2.550	0.30
DQ5	51 (26)	69 (27)	1.027	0.92
DQ6	24 (12)	44 (17)	1.960	0.19
DQ7	93 (47)	112 (43)	0.838	0.39

* Bonferroni p=0.005.

† Bonferroni p=0.025.

Table 5 The commonest* HLA-DRB1 alleles in Greek patients with scleroderma and controls. Results are shown as No (%) alleles

DRB1 alleles	Scleroderma (n=98) Alleles (n=196)	Controls (n=130) Alleles (n=260)	Odds ratio	Fisher's p value†
0101	6 (3)	16 (6)	2.077	0.18
0102	5 (3)	6 (2)	0.902	1.00
0301	13 (7)	16 (6)	0.923	0.85
0403	7 (4)	7 (3)	0.747	0.60
0404	0 (0)	7 (3)	—	0.0215
0701	9 (5)	19 (7)	1.638	0.25
1001	4 (2)	6 (2)	1.134	1.00
1101	10 (5)	18 (7)	1.383	0.56
1104	70 (36)	45 (17)	0.376	<0.0001
1301	5 (3)	15 (6)	2.339	0.11
1302	2 (1)	7 (3)	2.684	0.31
1401	3 (2)	10 (4)	2.573	0.166
1501	16 (8)	8 (3)	0.351	0.019
1502	1 (0.5)	10 (4)	7.800	0.028
1601	19 (10)	38 (15)	1.595	0.152
1602	4 (2)	6 (2)	1.134	1.00

*The alleles with frequencies higher than 5% either in the patient or the control group are presented.

† Bonferroni p=0.0017.

Table 6 The commonest* DQA1 and DQB1 alleles in Greek patients with scleroderma and controls. Results are shown as No (%) of alleles

Alleles	Scleroderma (n=98) Alleles (n=196)	Controls (n=130) Alleles (n=260)	Odds ratio	Fisher's p value
DQA1†				
0101	14 (7)	33 (13)	1.890	0.062
0102	43 (22)	55 (21)	0.954	0.91
0103	8 (4)	20 (8)	1.958	0.12
0104	6 (3)	5 (2)	0.620	0.54
0201	10 (5)	19 (7)	1.466	0.44
0301	16 (8)	30 (12)	1.467	0.27
0401	5 (3)	4 (2)	0.596	0.51
0501	90 (46)	92 (35)	0.6450	0.0263
DQB1‡				
0201	23 (12)	32 (12)	1.056	0.89
0301	75 (38)	87 (33)	0.811	0.32
0302	13 (7)	21 (8)	1.237	0.59
0402	5 (3)	2 (1)	0.296	0.14
0501	18 (9)	25 (10)	1.052	1.00
0502	31 (16)	34 (13)	0.800	0.42
0503	2 (1)	10 (4)	3.880	0.07
0601	2 (1)	8 (3)	3.080	0.20
0602	9 (5)	10 (4)	0.831	0.81
0603	10 (5)	17 (7)	1.301	0.55
0604	2 (1)	8 (3)	3.080	0.20

*The alleles with frequencies higher than 5% either in the patient or the control group are presented.

† Bonferroni p=0.005.

‡ Bonferroni p=0.003.

(25%)) than in ISSc (13/124 (10%)) or dSSc (7/114 (6%)) patients (p=0.043) (table 3).

ASSOCIATION OF AUTOANTIBODIES WITH ORGAN DISEASE

The patients were subdivided into positive and negative groups for each of the autoantibody specificities described previously, independently of scleroderma variants, and the association of each autoantibody specificity with organ disease was examined. Pulmonary disease, either of the interstitial pulmonary fibrosis type, or isolated pulmonary hypertension, occurred in 6/33 (18%) of the ACA positive and in 93/186 (50%) of the ACA negative patients (p=0.0006). Specifically, interstitial fibrosis occurred in 9% of ACA positive and 49% of ACA negative patients (p<0.0001); therefore ACA are negatively associated with pulmonary disease, in general, and with interstitial pulmonary fibrosis, in particular. However, isolated pulmonary hypertension occurred in 3/33 (9%) of ACA positive and 2/186 (1%) of ACA negative patients (p=0.025).

Anti-topo I positive patients tend to develop pulmonary disease (either of the interstitial fibrosis type or isolated pulmonary hypertension) more commonly (49/93 (53%)) than anti-topo I negative patients (50/126 (40%)) (p=0.074). When interstitial pulmonary fibrosis was examined, it occurred in 48/93 (52%) of anti-topo I positive and 46/126 (37%) of anti-topo I negative patients (p=0.028). Isolated pulmonary hypertension occurred in 1/93 (1%) of anti-topo I positive and 4/126 (3%) of anti-topo I negative patients.

Fifty four of the anti-topo I positive patients (57%) were positive for oesophageal disease compared with 58/143 (40%) of anti-topo I negative patients (p=0.014). Raynaud's phenomenon occurred in most patients (table 2). In addition, digital pitting scars, ulceration, or gangrene occurred in 65/94 (69%) of anti-topo I positive patients compared with 75/143 (52%) of anti-topo I negative patients (p=0.015). Other autoantibodies were not associated with major organ disease. The disease of other major organs such as the kidney and the heart was not associated with certain autoantibody specificities.

HLA ASSOCIATIONS WITH SSC

DNA was extracted from 98 Greek patients with SSc and 130 healthy Greek subjects as controls. The DRB1, DQA1, DQB1, and DPB1 alleles were identified. It was shown that the disease was associated with HLA-DR5 (DR11) (table 4). To explore further which of the DR11 associated alleles was particularly associated with SSc, comparisons were made between patients and controls for 29 different DRB1 alleles with the appropriate corrections (Bonferroni p=0.0017) (table 5). Furthermore, the two groups were compared for 10 different DQA1 alleles, 16 different DQB1 alleles, and 26 different DPB1 alleles (tables 6 and 7). It was shown that the DRB1*1104 allele occurs more commonly in patients (36%) than in controls (17%)

Table 7 The commonest* HLA-DPB1 alleles in Greek patients with scleroderma and controls. Results are shown as No (%) alleles

DPB1 alleles†	Scleroderma (n=96) Alleles (n=192)	Controls (n=130) Alleles (n=260)	Odds ratio	Fisher's p value
0201	39 (20)	58 (22)	1.126	0.60
0301	13 (7)	22 (8)	1.273	0.50
0401	57 (30)	83 (32)	1.111	0.68
0402	40 (21)	43 (17)	0.753	0.27
1001	3 (2)	6 (2)	1.488	0.74
1301	16 (8)	10 (4)	0.440	0.064
1401	2 (1)	7 (3)	1.040	0.31
1701	2 (1)	7 (3)	2.630	0.31

*Alleles with frequencies higher than 5% either in the patient or the control group are presented.

†Bonferroni p=0.0019.

Figure 1 Estimated survival curves in patients with scleroderma and controls matched for age and sex (general population).

($p<0.0001$). Other DR or DQ or DP alleles were not associated with the disease. When the patients were subdivided into SSc subgroups it was shown that the frequency of the DRB1*1104 allele tended to be higher in patients with dSSc (72%) than in patients with lSSc (51%) or overlap syndrome (38%) ($p=0.056$).

ASSOCIATION OF HLA ALLELES WITH AUTOANTIBODY SPECIFICITIES

The patients positive (either homozygotes or heterozygotes) for a particular HLA allele constituted the positive group for this allele and the remaining patients the negative group. Anti-topo I positive patients were 66% of the HLA-DRB1*1104 positive patients and only 17% of the HLA-DRB1*1104 negative patients ($p<0.001$). On the contrary, ACA positive patients were only 3.5% of the HLA-DRB1*1104 positive patients and 31% of the HLA-DRB1*1104 negative patients ($p<0.001$). The above results indicated a significant association between the HLA-DRB1*1104 allele and anti-topo I reactivity and significant lack of association of the same allele with ACA reactivity. No other (positive or negative) associations were found between particular HLA alleles and autoantibodies.

SURVIVAL AND REASONS FOR DEATH AMONG THE PATIENTS WITH SSC

Seven patients died during follow up, two in the lSSc group and five in the dSSc group. The reasons for death were as follows: pulmonary hypertension (two patients), renal crisis (two), heart and lung failure (one), heart disease with ventricular arrhythmia during the course of severe gastroenteritis (one), and unknown

cause (one). One of the patients who died from renal crisis, died six hours after admission from the emergency department.

No difference in death rates was detected between the two SSc groups ($p=0.26$). Time to death ranged from less than a year to four years after the first visit and up to 23 years after the first symptom. The estimated survival probability four years after the first visit was 94.8%. Figure 1 presents the Kaplan-Meier survival curve for the patients with scleroderma in our cohort, together with the survival probability for controls matched for age and sex from the general population.

Discussion

The profile of Greek patients with SSc, with particular emphasis on the prevalence of major organ disease, serological characteristics, association with HLA class II alleles, associations of clinical with serological findings, survival rate, and reasons for death, is presented.

The disease affects mainly women in their fourth to fifth decade of life with a female to male ratio of 6.7:1, which is similar to that (6.4:1) found among consecutive Japanese patients.²¹ Differences exist in the literature (from older to recent reports) about the female to male ratio, which ranges from 2.9:1 to 9:1.^{12 31-34} Two explanations can be offered for these discrepancies: (a) an increased prevalence of scleroderma among women occurs with time, therefore environmental pressures predisposing to disease should be carefully evaluated; (b) the designs of the studies differ greatly. Indeed, hospital based surveys,¹² descriptive analysis of patients referred to tertiary care centres,³² or analysis of consecutive patients examined in a single hospital^{33 34} are characteristic examples of the different patient selection strategies used.

The prevalence of various autoantibodies in the disease variants remains controversial. A comparison of serological findings among different studies is difficult because in the older studies³⁵ the patients with SSc were classified as those with progressive systemic sclerosis (PSS)³⁶ and those with CREST³⁷; these disease variants are not identical to the recently proposed dSSc and lSSc variants, respectively.

ACA were found exclusively among patients with lSSc in this study with a prevalence of 34%. The prevalence of ACA in lSSc (or patients with CREST in the older studies) ranged from 28% to 49%.^{21 32 35 38-41} It was realised that the prevalence of ACA was more common in white subjects, whereas the prevalence of anti-topo I was more common in non-white subjects, such as African Americans¹⁷ or Thais.⁴² Therefore, the low prevalence of ACA in a previous study³⁹ might be related to the fact that different racial groups were included.

It is reported that a limited number of patients with dSSc (or with PSS patients in older studies) also possess ACA in their sera. The prevalence of ACA in patients with dSSc was found to be from 0% to 14%.^{21 35 38-44}

It can be concluded that ACA occur in 1/3 to 1/2 patients with lSSc, with a tendency to be higher in white populations; ACA occur also in

patients with dSSc but with a prevalence no higher than 8% as found in most of the studies mentioned.

Antibodies to topo I occur mainly in patients with dSSc, but the prevalence found varies from 18%³⁹ to 77%,⁴¹ ranging widely between studies.^{21 32 35 39 40-42 45} Our study reconfirmed previous results obtained by evaluating our first series of patients⁴³ and suggests that nearly 3/5 of our patients with dSSc are anti-topo I positive. This prevalence is lower than that described in Japanese,⁴¹ Thais⁴² or black subjects,¹⁷ but seems to be higher than that described in Anglo-Saxon populations.^{14 32 35}

A low prevalence of anti-topo I has been described in patients with lSSc, ranging from a complete absence³⁹ to 25%.⁴³ A reasonable explanation for such discrepancies is that the disease in a limited number of patients with lSSc with anti-topo I evolves slowly to the dSSc; therefore such patients are classified either as the lSSc or dSSc variant depending on the duration of the follow up and their features at the time of evaluation. The prevalence of lSSc anti-topo I positive patients in this study was 21%; this is comparable with the results found by Kuwana *et al.*²⁴ Steen *et al.*,³² and Satoh *et al.*⁴¹ and higher than the results described by others.^{32 39 40}

Our study showed that lung, oesophageal, heart, and peripheral vessel disease occurred more commonly in dSSc than in lSSc, whereas kidney disease and, particularly renal crisis, was a rare complication. Pulmonary disease defined as interstitial pulmonary fibrosis or isolated reduction of TLCO occurred in 52% of our patients; a result comparable with that found in other patient series.^{9 21 32 35 39 40} Oesophageal disease is slightly underreported compared with other studies, probably owing to the retrospective nature of our work. During follow up the disease was based on clinical grounds and when symptoms were present it was further assessed by manometry or radiographic evaluation. The prevalence of cardiac disease was found to be 14%, which is comparable with that found by others.^{9 21 32 35 39 40} Kidney disease occurred in 4% of our patients; this prevalence is comparable with that found by Kuwana *et al.*²⁴ and Reimer *et al.*,⁹ but it is slightly lower than that described by Steen *et al.*³² and definitely lower than that described in other European or American reports.^{35 39 40} Joint disease, as it was described by others,³⁵ occurred in 27% of our patients, which is in contrast with other reports describing largely different figures ranging from 6%³⁹ to 60%.³² New information was added to the patients' files at every visit. Therefore the files were available for longitudinal analysis. We chose to present the incidence of oesophageal, cardiac, and pulmonary disease per 100 patient-years.

The association of ACA with lSSc and the association of anti-topo I with dSSc have been already confirmed in many studies.^{21 32 35 38 43} ACA were negatively associated with pulmonary fibrosis, whereas they were associated with isolated pulmonary hypertension in this study. Among Japanese patients with SSs, pulmonary hypertension was associated with anti-

U1RNP,²¹ which occur in 27% of Japanese patients with SSs; such antibodies were present in 7.5% of our patients with SSs. Anti-U1RNP, in general, are present rarely among European populations.⁴⁵

A positive association of ACA with telangiectasis, calcinosis, women,²⁷ primary biliary cirrhosis,²¹ and oesophageal hypomotility,³⁸ and a negative association with pulmonary fibrosis^{21 32 35 38} have been described and were reconfirmed in this study.

Anti-topo I have been associated with pulmonary fibrosis and digital ischaemia,^{21 32 35 43} and these data also were reconfirmed here.

The association of HLA alleles with the disease is controversial. Several explanations exist for the discrepancies found between various studies: (a) the products of the HLA system were serologically defined in previous studies, whereas in recent studies the HLA alleles were determined by PCR; (b) various HLA alleles differ in their frequencies among different ethnic groups; (c) particular HLA alleles were associated with the disease as a whole,^{20 46-49} by some authors, whereas others found an association between particular HLA alleles and some disease related features.^{19 20 48-50} However, there is some agreement that HLA-DR1 or HLA DR5, or both, are associated with the disease.^{46-49 51 52} In one study the disease was associated with HLA-DQA2, which is in linkage disequilibrium with DR3/DR11.²⁰ The combination of DR3/DRw52 was associated with pulmonary fibrosis,¹⁹ and DRw52 has been associated with pulmonary hypertension by others.⁴⁸

The mortality rate of Greek patients with SSs is lower than that described in American reports.¹⁴ Patient selection strategies play an important part in the discrepancies in mortality rates seen in various studies, but the susceptibility of African Americans for rather severe disease forms contributes to their high mortality rates. However, a five year survival of 50%¹⁴ probably reflects an overestimation of the death rate described in patients selected by a tertiary care centre. Reports from the UK,¹⁵ South Africa,¹⁶ and India¹⁸ describe mortality rates closer to those found in our study. The standardised mortality rate was found to be 0.40/100 000 in a report from Australia.⁵³ The five and 10 year survival rates were 86% and 69%, respectively, in Swedish patients, and there was a 4.6-fold increased risk of death compared with the general population.⁵⁴ A 10 year survival of 85% after the first symptom was also reported in Spain.⁵⁵ The standardised mortality ratio was found to be 2.9 in a report from Denmark⁵⁶ and 4.69 in a report from Canada.⁵⁷ In a study from Japan a 93.7% five year survival and 82% 10 year survival, as calculated from the first symptom, were reported.⁵⁸

The study design in its relation to survival measurements is not comparable between studies. Other studies report five and 10 year survival, while yet others calculate standardised mortality rates, which reflect a comparison of survival between the patients and the general

population. To perform the above measurements, other studies consider duration of patient follow up as the time from the first symptom, whereas others consider the follow up as starting after the first visit to the reporting centre. We used the latter approach, which helped us to analyse information based on our observations. Regardless of the contradictions, the above studies indicate that increased mortality occurs in patients with SSc, but this tends to be lower than that reported in previous studies. The mean follow up in our cohort was less than three years, which might explain the low standardised mortality rate even compared with the recent studies mentioned above.

Most of the patients of our cohort appeared in the outpatient clinic seeking primary care owing to their symptoms rather than by referral from other hospitals or doctors; thus the evolution of their disease reflects largely the evolution of SSc in the general population. The fact that a clinical chart was always completed at the first patient visit, even in the emergency outpatient department, eliminates the possibility that short term survivors from those attending our hospital were missed in this study. However, short term survivors may be missed in community hospitals before their referral to us; therefore, a national, or even international, survey on scleroderma mortality is mandatory.

A significant association of SSc with the HLA class II DRB1*1104 allele was found in this study. Furthermore, the DRB1*1104 allele was associated preferentially with the diffuse form of the disease; it was negatively associated with the presence of ACA and significantly associated with the presence of anti-topo I. It has been found previously that among white subjects with anti-topo I, the frequency of DRB1*1101 and DRB1*1104 alleles is high.⁵²⁻⁵⁹ DRB1*1104 possess the ⁶⁷FLEDR⁷¹ sequence in the DRB1 domain, which is highly associated with antibodies to one of the major epitopes of topo I.⁶⁰ Our finding on HLA-DRB1*1104 association with anti-topo I is further corroborated by a recent report describing a strong association between them.⁶¹ The same association was recently reported in an American study.⁶²

In conclusion, SSc in Greek patients is characterised by a low prevalence of renal disease, and a relatively high survival rate, despite a prevalence of anti-topo I, which are associated with an advanced disease course, similar to that found in other populations. The disease is associated with the HLA-DRB1*1104 allele which also seems to control the development of anti-topo I.

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