Combined seronegativity in Sjögren's syndrome

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ABSTRACT

Objective. *To describe the clinical spectrum of Sjögren's syndrome (SS) patients with combined seronegativity.*

Methods. *From a multicentre study population of consecutive SS patients fulfilling the 2016 ACR-EULAR classification criteria, patients with triple seronegativity [anti-Ro/SSA(-), anti-La/SSB(-), RF(-) and ANA(+)] and quadruple seronegativity [anti-Ro/SSA(-), anti-La/SSB(-), RF(-) and ANA(-)] were identified retrospectively. Both groups were matched in an 1:1 ratio with 2 distinct control SS groups: i) classic anti-Ro/SSA seropositive patients [SS(+)] and ii) classic anti-Ro/SSA seropositive patients with negative rheumatoid factor [SS(+)/RF(-)] to explore their effect on disease expression. Clinical, laboratory and, histologic features were compared. A comparison between triple and quadruple seronegative SS patients was also performed.*

Results. *One hundred thirty-five SS patients (8.6%) were identified as triple seronegative patients and 72 (4.5%) as quadruple. Triple seronegative patients had lower frequency of peripheral nervous involvement (0% vs. 7.2% p=0.002) compared to SS(+) controls and lower frequency of interstitial renal disease and higher prevalence of dry mouth than SS(+)/RF(-) controls. Quadruple seronegative patients presented less frequently with persistent lymphadenopathy (1.5% vs. 16.9 p=0.004) and lymphoma (0% vs. 9.8% p=0.006) compared to SS(+) controls and with lower prevalence of persistent lymphadenopathy (1.5% vs. 15.3% p=0.008) and higher frequency of dry eyes (98.6% vs. 87.5% p=0.01) and autoimmune thyroiditis (44.1% vs. 17.1% p=0.02) compared to SS(+)/RF(-) SS controls. Study groups comparative analysis revealed that triple seronegative patients had higher frequency of persistent lymphadenopathy and lymphoma, higher focus score and later age of SS diagnosis compared to quadruple seronegative patients.*

Conclusion. *Combined seronegativity accounts for almost 9% of total SS population and is associated with a milder clinical phenotype, partly attributed to the absence of rheumatoid factor.*

Introduction

Sjögren's syndrome (SS) is accompanied by plethora of autoantibodies as a result of B cell aberrant activation (1, 2), with anti-Ro/SSA, anti-La/SSB, rheumatoid factor (RF) and antinuclear antibodies (ANA) being the most frequently encountered (3, 4). Anti-Ro/SSA antibody, is present in 50-75% of SS patients and in approximately half of them, anti-La/SSB antibody is also detected (5). Anti-La/SSB antibodies almost always coexist with anti Ro/SSA and less than 2% of SS patients are anti-La/SSB monopositive, with a phenotype ranging between seronegative and seropositive patients (6, 7). The prevalence of rheumatoid factor reaches approximately 50% in SS and have been recognised as lymphoma predictor (8, 9). Other autoantibodies have been also reported to define unique clinical phenotypes of SS but are detected in low prevalence (1). Preceding studies have explored the phenotype of partly seronegative patients (defined as anti-Ro/SSA and anti-La/SSB negative antibodies) *versus* seropositive controls (defined as positive for anti-Ro/SSA with or without anti-La/SSB antibodies), exhibiting differences regarding the age at SS diagnosis, sicca manifestations, specific extraglandular manifestations, and lymphoma (10, 11). In these studies, however, RF and ANA antibodies differed between study groups, obscuring the true effect of anti-Ro/SSA and/

Table I. Comparison of clinical and laboratory features between 3pl negative patients and the two controls groups.

Feature/Clinical manifestation	3PL Negatives n=135	Ro (+) Controls n=135	*p*-value	RF (-), Ro (+) Controls n=135	*p*-value
Demographics					
Median age at disease diagnosis, (range)	60, (26-80)	60, (26-79)	0.97	60, (28-83)	0.81
Median disease duration from SS diagnosis to last follow-up, (range)	3, (0-28)	3, (0-25)	0.75	3, (0-28)	0.86
Glandular and non-specific manifestations					
Dry mouth	96.2% (128/133)	91.8% (1324/135)	0.20	88% (118/134)	**0.02**
Dry eyes	94.8% (128/135)	91.8% (124/135)	0.46	90.3% (122/135)	0.24
Salivary gland enlargement	22.3% (30/134)	24.6% (33/134)	0.77	14.1% (19/134)	0.11
Raynaud's phenomenon	34.3% (44/128)	31.7% (39/123)	0.75	30.6% (38/124)	0.61
Arthralgias	53.3% (71/133)	56.7% (76/134)	0.58	60% (81/135)	0..33
Arthritis	15.5% (19/122)	13.6% (16/117)	0.81	12.9% (15/116)	0.69
Extraepithelial manifestations					
Glomerulonephritis	0.7% (1/132)	0.7% (1/135)	1	2.2% (3/135)	0.62
Interstitial lung disease	7.7% (10/129)	7.8% (10/127)	0.84	4.8% (6/125)	0.47
Autoimmune hepatitis	0% (0/111)	0.8% (1/112)	1	0.9% (1/104)	0.48
Peripheral nervous disease	0% (0/124)	7.2% (8/110)	**0.002**	2.5% (3/116)	0.11
Palpable purpura	3.7% (5/135)	9.6% (13/135)	0.08	1.4% (2/135)	0.44
Persistent lymphadenopathy	12.5% (16/127)	11.7% (13/111)	0.99	8.8% (11/125)	0.44
Periepithelial manifestations					
Tubulointerstitial nephritis	0% (0/132)	1.5% (2/133)	0.49	5.2% (7/134)	**0.01**
Small airway disease	7.2% (9/124)	7.7% (9/116)	0.92	7.7% (9/116)	0.92
Primary biliary cholangitis	3.7% (5/135)	1.4% (2/135)	0.44	1.4% (2/134)	0.44
Autoimmune thyroiditis	30.5% (26/85)	29.6% (24/81)	0.97	28.3% (23/81)	0.88
Focus score (range)	2 (1-9)	1.83 (0-12)	0.28	2 (0,25-12)	0.06
Serology					
Rheumatoid factor	0% (0/135)	62.2% (84/135)	<0.0001	0% (0/135)	1
Anti-Ro	0% (0/135)	89.6% (135/135)	<0.0001	100% (135/135)	<0.0001
Anti-La	0% (0/135)	40% (54/135)	<0.0001	30.3% (41/135)	<0.0001
LOW C4	23.1% (28/121)	23.1% (38/116)	0.13	23.3% (28/120)	0.90
Monoclonality	7.2% (9/124)	11% (13/118)	0.42	7.5% (9/120)	0.86
Cryoglobulinaemia	6.8% (6/88)	10.2% (8/78)	0.62	6.4% (5/77)	0.81
Anti-nuclear antibody	100% (135/135)	100% (135/135)	1	100% (135/135)	1
Lymphoma	5.9% (8/135)	10.3% (14/135)	**0.26**	1.4% (2/135)	0.1

or anti-La/SSB antibodies on disease phenotype. Recently, it was shown that the absence of SS associated autoantibodies is related to fewer haematologic malignancies, although lymphomas were not included in the analysis (12). Prompted by these findings, we sought to explore the clinical landscape of SS patients with autoantibody paucity and investigate the effect of RF on the clinical expression of the disease.

Patients and methods

Study population included 1723 consecutive SS patients who fulfilled the 2016 EULAR/ACR criteria and were followed between May 1984 and January 2021, in 4 clinical centres ([Universities of Pisa, Athens, Harokopio and Ioannina]. Patients with unknown autoantibody profile were excluded. Two study groups were identified: a) patients with triple seronegativity [anti-Ro/SSA (-), anti-La/SSB (-), RF(-) and ANA (+)](3pl) and b) patients with quadruple seronegativity [anti-Ro/SSA (-), anti-La/SSB (-), RF(-) and ANA (-)](4pl). Each study group was compared with 2 distinct SS control groups, matched according to gender, age at disease diagnosis and disease duration from SS diagnosis to last follow-up, in a 1:1 ratio, and in terms of cumulative clinical, laboratory and histologic features: i) randomly chosen classic anti-Ro/SSA seropositive SS controls, independently of the RF and anti-La/SSB status [anti-Ro/SSA(+), ANA(+), anti-La/SSB (+or-), RF (+or-)] [SS(+)] and ii) classic seropositive anti-Ro/SSA SS controls with negative RF [anti-Ro/SSA (+), ANA (+), anti-La/SSB (+or-), and RF(-)] [SS(+)/RF(-)] to investigate the effect of RF on clinical phenotype of the disease and especially lymphoma. All data were collected retrospectively from medical records, following a common prespecified reference model, as part of the HarmonicSS project. Systemic organ involvement and all clinical variables were defined as previously described by the ESSDAI domains and glandular dryness according to EULAR validated questionnaires (13, 14). Salivary gland biopsies were evaluated by 2 independent and highly experienced in SS pathologists. Autoantibodies were detected in all centres as part of standard of care accordingly. The study was approved by all local ethical committees after obtaining patients' informed consent and in compliance with general data

Table II. Comparison of clinical and laboratory features between 4pl negative patients and the two controls groups.

Feature/Clinical manifestation	4PL Negatives n=72	Ro (+) Controls n=72	*p*-value	RF (-), Ro (+) Controls n=135	*p*-value
Demographics					
Median age at disease diagnosis, (range)	56, (11-74)	56, (10-77)	0.91	56, (10-76)	0.89
Median disease duration from SS diagnosis to last follow-up, (range)	4, (0-24)	4, (0-23)	0.96	3, (0-21)	0.49
Glandular and non-specific manifestations					
Dry mouth	94.3% (67/71)	94.2% (67/71)	1	88% (64/72)	0.36
Dry eyes	98.6% (71/72)	91.6% (66/72)	0.11	87.5% (63/72)	**0.01**
Salivary gland enlargement	16.6% (12/72)	30.5% (22/72)	0.07	20.8% (15/72)	0.66
Raynaud's phenomenon	25.3% (16/63)	27.9% (19/68)	0.98	27.2 (18/66)	0.96
Arthralgias	54.1% (39/72)	61.1% (44/72)	0.61	58.3% (42/73)	0.73
Arthritis	6.4% (4/62)	18.7% (12/64)	0.06	9.8% (6/61)	0.52
Extraepithelial manifestations					
Glomerulonephritis	1.4% (1/71)	2.8% (2/71)	1	1.4% (1/71)	1
Interstitial lung disease	3% (2/65)	7.1% (5/70)	0.44	1.5% (1/66)	0.61
Autoimmune hepatitis	0% (0/62)	1.9% (1/52)	0.45	1.7% (1/57)	0.47
Peripheral nervous disease	3.2% (2/61)	3% (2/65)	1	3.3% (2/60)	1
Palpable purpura	4.1% (3/72)	12.5% (9/72)	0.13	0% (0/72)	0.24
Persistent lymphadenopathy	1.5% (1/65)	16.9% (11/65)	**0.004**	15.3% (10/65)	**0.008**
Periepithelial manifestations					
Tubulointerstitial nephritis	2.8% (2/71)	2.7% (2/72)	1	1.4% (1/71)	1
Small airway disease	5% (3/60)	7.4% (5/67)	0.72	8% (5/62)	0.71
Primary biliary cholangitis	1.3% (1/72)	1.3% (1/72)	1	1.4% (1/72)	1
Autoimmune thyroiditis	44.1% (19/43)	29.0% (9/31)	0.31	17.1% (6/35)	**0.02**
Focus score (range)	1.1 (1-4)	2 (0-8.7)	0.07	1.33 (0-12)	0.83
Serology					
Rheumatoid factor	0% (0/72)	47.2% (34/72)	<0.0001	0% (0/72)	1
Anti-Ro	0% (0/72)	100% (72/72)	<0.0001	100% (72/72)	<0.0001
Anti-La	0% (0/72)	47.2% (34/72)	<0.0001	34.7% (25/72)	<0.0001
LOW C4	23.3% (14/60)	39.0% (25/64)	0.09	33.8% (21/62)	0.27
Monoclonality	3.2% (2/62)	7.4% (5/67)	0.44	8% (5/62)	0.43
Cryoglobulinaemia	6.8% (3/44)	13.8% (5/36)	0.45	13.5% (5/37)	0.45
Anti-nuclear antibody	0% (0/72)	100% (72/72))	<0.0001	100% (70/72)	<0.0001
Lymphoma	0% (0/72)	9.8% (7/71)	**0.006**	1.3% (1/72)	1

protection regulations (GDPR) of the European Union. Statistical analysis for categorical data was performed by Fisher exact test or chi-square test and numerical data with the Mann-Whitney or t-test using Python 3.6.

Results

Patients' characteristics

Final population comprised of 1569 patients with full SS related autoantibody profile; 135 triple seronegative patients and 72 quadruple seronegative patients were identified, constituting 8.6% and 4.6% respectively. Seronegative SS patients from each study group were compared with i) 135 and 72 classic anti-Ro/SSA seropositive SS control patients respectively [SS(+)], of whom 84 (62.2%) and 34 (47.2%) had positive RF respectively and ii) 135 and 72 SS controls respectively, with RF(-)/anti-Ro/SSA seropositivity [SS(+)/RF(-)]. The median age at SS diagnosis was 60 years old (range: 26-80) for 3pl negative patients and 56 years old (range: 11-74) for 4pl negative patients while disease duration was 3 years for both groups (range: 0-28 and 0-24, respectively). Each study group included five male patients. The demographic features of SS control groups are presented in Tables I and II.

Comparison of triple and quadruple negative patients with distinct SS control groups

- *Anti-Ro/SSA(+) SS controls independently of RF*

Demographic, clinical, laboratory and histologic features were compared between the two study groups and their respective anti-Ro/SSA(+), ANA(+) RF(+or-) SS control groups [SS(+)]. Demographic characteristics were similar between comparing groups (Table I). A statistically significantly lower frequency of peripheral nervous involvement (0% *vs*. 7.2% *p*=0,002) was observed in 3pl negative SS patients compared to SS(+) controls. Similarly, purpura and lymphoma were less prevalent among 3pl negative patients, without though reaching statistical significance (Table I). Quadruple negative patients presented less frequently with persistent lymphadenopathy (1.5% *vs*. 16.9 *p*=0.004) and lymphoma (0% *vs*. 9.8% *p*=0.006) compared to SS(+) controls (Table II). Similarly, 4pl negative SS patients developed less often salivary gland enlargement, low C4 hypocomplementaemia, arthritis, pur-

pura and cryoglobulinaemia, without statistically significant difference.

- *SS controls with RF negativity and anti-Ro/SSA positivity*

Demographic, clinical, laboratory and histologic features were compared between the two study groups and SS(+)/RF(-) control groups. Demographic characteristics were similar between comparing groups (Table I). A statistically significantly higher frequency of dry mouth was found on 3pl negative patients compared to controls (96.2% *vs*. 88% p=0.02) whereas SS(+)/RF(-) control patients displayed higher prevalence of interstitial renal disease (0% *vs*. 5.2% p=0.01). Quadruple negative patients presented more frequently with dry eyes (98.6% *vs*. 87.5% p=0.01) and autoimmune thyroiditis (44.1% *vs*. 17.1% p=0.02) while their SS(+) RF(-) SS controls displayed a stronger association with persistent lymphadenopathy (1.5% *vs*. 15.3% p=0.008) (Table II).

Comparison of triple and quadruple negative patients

Demographic, clinical, laboratory and histologic features were compared between 3pl and 4pl negative patients. The median disease duration of comparing groups was similar, while 4pl negative patients showed an earlier age at SS diagnosis than 3pl negative patients (Table III). A statistically significantly higher frequency of persistent lymphadenopathy (12.5% *vs*. 1.5% p=0.01), minor salivary gland biopsy focus score (2% *vs*. 1.1% p=0.01) and lymphoma (5.9% *vs*. 0% p=0.05) was associated with 3pl negative patients. Quadruple negative patients displayed an increased prevalence of autoimmune thyroiditis and peripheral nervous disease, without reaching a statistically significant difference (Table III).

Table III. Comparison of clinical and laboratory features between 3pl negative patients and 4pl negative patients.

Feature/ Clinical manifestation	3PL Negatives n=135	4PL Negatives n=72	p-value
Demographics			
Median age at disease diagnosis, (range)	60, (26-80)	56, (11-74)	**0.02**
Median disease duration from SS diagnosis to last follow-up, (range)	3, (0-28)	4, (0-24)	0.70
Glandular and non-specific manifestations			
Dry mouth	96.2% (128/133)	94.3% (67/71)	0.49
Dry eyes	94.8% (128/135)	98.6% (71/72)	0.26
Salivary gland enlargement	22.3% (30/134)	16.6% (12/72)	0.42
Raynaud's phenomenon	34.3% (44/128)	25.3% (16/63)	0.35
Arthralgias	53.3% (71/133)	54.1% (39/72)	0.79
Arthritis	15.5% (19/122)	6.4% (4/62)	0.09
Extraepithelial manifestations			
Glomerulonephritis	0.7% (1/132)	1.4% (1/71)	1
Interstitial lung disease	7.7% (10/129)	3% (2/65)	0.34
Autoimmune hepatitis	0% (0/111)	0% (0/62)	1
Peripheral nervous disease	0% (0/124)	3.2% (2/61)	0.10
Palpable purpura	3.7% (5/135)	4.1% (3/72)	1
Persistent lymphadenopathy	12.5% (16/127)	1.5% (1/65)	**0.01**
Periepithelial manifestations			
Tubulointerstitial nephritis	0% (0/132)	2.8% (2/71)	0.34
Small airway disease	7.2% (9/124)	5% (3/60)	0.75
Primary biliary cholangitis	3.7% (5/135)	1.3% (1/72)	0.66
Autoimmune thyroiditis	30.5% (26/85)	44.1% (19/43)	0.21
Focus score (range)	2 (1-9)	1.1 (1-4)	**0.01**
Serology			
Rheumatoid factor	0% (0/135)	0% (0/72)	1
Anti-Ro	0% (0/135)	0% (0/72)	1
Anti-La	0% (0/135)	0% (0/72)	1
LOW C4	23.1% (28/121)	23.3% (14/60)	0.87
Monoclonality	7.2% (9/124)	3.2% (2/62)	0.34
Cryoglobulinaemia	6.8% (6/88)	6.8% (3/44)	1
Anti-nuclear antibody	100% (135/135)	0% (0/72)	<0.0001
Lymphoma	5.9% (8/135)	0% (0/72)	**0.05**

Discussion

Double anti-Ro/SSA and anti-La/SSB seronegativity has been a subject of intense clinical research (10, 11). However, little is known about triple and quadrable combined seronegativity, enclosing the significant contribution of RF as the crossroad among B cell hyperactivity, monoclonality and lymphomagenesis, in the clinical phenotype of the disease. Our study gathers some unique features: a) it is conducted in 4 highly specialised clinical centres for SS from 2 different countries, b) integrated data are manually harmonised based on a common reference model, and c) it is focused on 3pl and 4pl seronegative SS patients for the first time in the literature, and d) two sequential comparisons have been performed with and without RF contribution, to explore their potential effect on clinical phenotype.

The first interesting findings of our study is the prevalence of patients with combined seronegativity reaching almost 9% of total SS population. This subset of patients is not negligible and is characterised by complete absence of autoantibodies, as typical serum biomarkers of autoimmunity. Second, it seems that both 3pl and 4pl negative patients adopt a milder clinical phenotype with less B cell mediated manifestations. Interestingly, 4pl negative patients have a tendency to present more frequently dry eyes, autoimmune thyroiditis and interstitial renal disease, especially when compared to SS(+)/RF(-) controls. These particular clinical manifestations, share as common underlying pathogenetic mechanism, the typical peri-pethelial inflammatory infiltration of the affected organs. This finding, in combination with the absence of autoantibodies, raises thoughts that 4pl negative SS patients might unleash strong local and systemic immunoregulatory mechanisms, capable of restricting the autoimmune response

confined to the epithelial structures, avoiding in this way generalised and wide-spread immune responses against ubiquitous self-antigens. Third, our study reveals that 3pl seronegative patients have a worse prognosis compared to 4pl with a higher probability of developing persistent lymphadenopathy and lymphoma combined with a higher salivary gland focus score (15). This finding is of clinical significance highlighting the fact that among seronegative patients those with positive antinuclear antibodies are expected to develop more severe disease manifestations as a consequence of a more generalised systemic autoimmune response. However, we should stress out that these patients were not matched, and our results may be affected by the confounding factor of age, that can alter the clinical trajectory of SS patients (16). Fourth, the similar prevalence of lymphoma between seronegative SS patients and SS(+)/RF(-) controls as opposed to SS(+) controls, could imply a central role of RF in the evolution towards lymphomagenesis. Indeed, RF have been previously proposed as independent lymphoma predictor (8). However, the clinical expression of the disease should not be interpreted only from the side of the effector arm (*e.g.* autoantibodies) but the counter immunoregulation should be also considered, and therefore not all RF+ SS patients are finally expected to develop a worse clinical phenotype. In the literature, only one study after our initial publication (17) has focused on 4pl negative SS patients, showing no differences between seropositive and seronegative patients, apart from hypergammaglobulinemia (12). In that study it was noteworthy the high proportion of 4pl negative SS patients, the omission of lymphoma as clinical feature, the tendency of these patients to develop autoimmune thyroiditis confirming our results and the absence of matched SS seropositive control groups, with and without RF(-) SS patients.

The present study is limited by the retrospective nature of the design, the relatively limited number of recruited triple and quadruple seronegative patients, the short period of follow up and the inherent physicians' hesitancy to perform further diagnostic work up for SS in suspicious individuals with negative autoantibody profile. In conclusion, SS patients with autoantibody paucity, display a mild clinical picture dominated by glandular and peri-epithelial manifestations while B cell symptoms are less apparent.

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