Anti-Ro52 and/or anti-Ro60 immune reactivity: autoantibody and disease associations

E. Zampeli[1], M. Mavrommati[2], H.M. Moutsopoulos[3], F.N. Skopouli[2,4]

[1]*Institute for Autoimmune Systemic and Neurological Diseases, Athens;* [2]*Department of Internal Medicine and Autoimmune Diseases, Euroclinic of Athens;* [3]*Academy of Athens;* [4]*Department of Nutrition and Dietetics, Harokopio University, Athens, Greece.*

Evangelia Zampeli, MD, PhD
Maria Mavrommati, MD
Haralampos M. Moutsopoulos, MD, FACP, FRCP (hc), Master ACR
Fotini N. Skopouli, MD, FRCP

Please address correspondence to:
Fotini N. Skopouli,
Department of Nutrition and Dietetics,
Harokopio University,
70 El. Venizelou Str,
17670 Athens, Greece.
E-mail: fskopouli@hua.gr

Received on October 8, 2019; accepted in revised form on January 8, 2020.

Clin Exp Rheumatol 2020; 38 (Suppl. 126): S134-S141.

Key words: anti-Ro52, anti-Ro60, Sjögren's syndrome, idiopathic inflammatory myopathies, systemic lupus erythematosus

Funding: the work was supported by HORIZON 2020, 731944 - HarmonicSS - H2020-SC1-2016-2017/h2020-SC1-2016-RTD, "HARMONIzation and integrative analysis of regional, national and international Cohorts on primary Sjögren's Syndrome (pSS) towards improved stratification, treatment and health policy making".

Competing interests: none declared.

ABSTRACT

Objective. *This study aims to characterise the clinical phenotype and autoantibody associations in an autoimmune population positive for anti-Ro52 and/or anti-Ro60 autoantibodies.*

Methods. *The sera of 508 individuals tested for autoantibody presence were found positive for anti-Ro52 and/or anti-Ro60. Medical records were available for 272 of them. Correlations of clinical, laboratory and other autoantibodies as well as disease phenotypes with the presence of anti-Ro52 and/or anti-Ro60 reactivity were examined.*

Results. *Combined serum anti-Ro52/anti-Ro60 reactivity was the most frequent one, mostly seen in Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE) patients. In these patients this reactivity strongly associated with anti-La and/or anti-dsDNA autoantibodies. SS patients with combined anti-Ro52/anti-Ro60 and anti-La reactivity had clinical and/or laboratory risk factors for lymphoma development. Solo anti-Ro52 reactivity was primarily found in idiopathic inflammatory myopathies (IIM), primary biliary cholangitis (PBC), rheumatoid arthritis (RA) and SS patients. Solo anti-Ro52 also associated with anti-Jo1 and anti-M2 autoantibodies and with interstitial lung disease (ILD) in a context of IIM-related lung injury. ILD patients with combined anti-Ro52/anti-Ro-60 reactivity were diagnosed mostly as RA and/or SS. Solo anti-Ro60 reactivity strongly correlated with oral ulcers and co-existed with autoantibodies to Sm and nRNP/Sm.*

Conclusion. *Testing for autoantibodies against both Ro peptides may guide diagnosis, classify clinical manifestations in disease entities and define prognosis in certain autoimmune disorders. A distinct weight could be given to the isolated anti-Ro specificities in the SS classification criteria.*

Introduction

Systemic autoimmune diseases can affect multiple organs and present with a wide variety of clinical and laboratory manifestations. Numerous autoantibodies have been associated with different clinical manifestations and distinct disease subgroups and their presence can guide diagnosis, prognosis, and in some cases, may direct therapeutic interventions (1).

Anti-Ro/SSA antibodies are among the most commonly detected autoantibodies in the routine screening for autoimmune diseases (2). Anderson *et al.* originally described the autoantigens which the autoantibodies recognise in salivary and lacrimal gland extracts from Sjögren's syndrome (SS) patients. The autoantigens were called "SjD" and "SjJ"(3). Subsequently, a name was given to these autoantigens derived partly from the initials of a systemic lupus erythematosus (SLE) patient ("Ro", Roland) (4) and thereafter partly from its association with SS, Ro (SSA) (5). Eventually, the molecular characteristics of this target antigen were identified, and the molecular masses were included in the nomenclature yielding the Ro60 and Ro52 auto-antigenic targets, which were shown to be different proteins coded by different cDNAs (6-8). Ro52 belongs to the family of tripartite motif proteins (TRIMs) and it is also denoted as TRIM21. It acts in the process of ubiquitination and although it is predominantly a cytoplasmic protein, it can translocate into the nucleus in a pro-inflammatory environment and regulate production of type-1 interferon (9). Ro60 protein on the other hand acts as quality-control for misfolded RNA. Defective RNA is recognised by Ro60 and appended for degradation (10, 11).

Antibodies to Ro52 and Ro60 have been associated with different autoimmune processes. Anti-Ro60 is found mainly in the sera of SLE and SS patients,

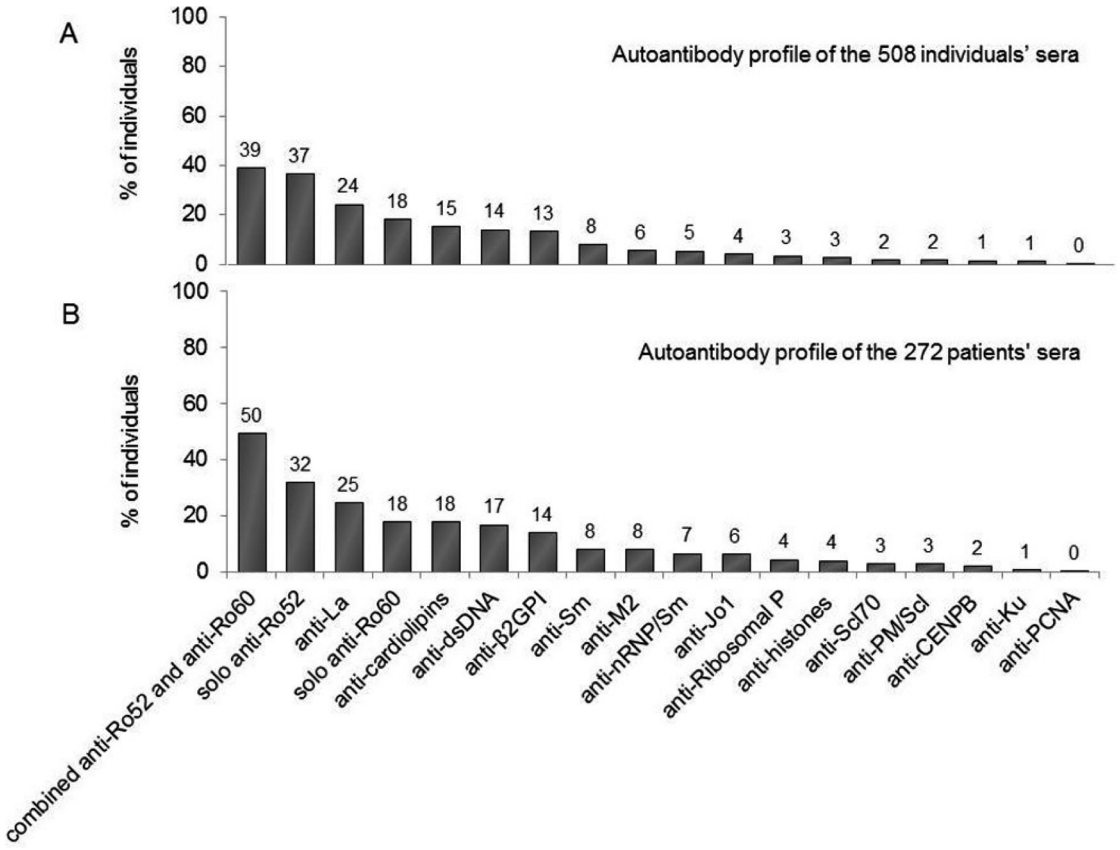

Fig. 1. Prevalence of autoantibodies in the entire cohort of 508 individuals **(A)** and in the 272 patients with available medical records **(B)**.

whereas anti-Ro52 shows a broader spectrum of disease associations (12, 13). More specifically, autoimmune clinical entities and conditions associated with anti-Ro52 are SS, interstitial lung disease (ILD) in systemic sclerosis (SSc) (14), autoimmune liver diseases (15), myositis and antisynthetase syndrome (16) as well as congenital heart block in neonatal lupus (17).

The aim of this retrospective, observational study was to analyse the clinical relevance and the disease phenotype of patients with anti-Ro52 and/or with anti-Ro60 autoantibodies as well as their associations with other autoimmune reactivity in a Greek Caucasian autoimmune patient population.

Methods

From May 2002 to December 2018 in the Immunology Laboratory of Euroclinic Hospital, Athens-Greece, the sera of 508 individuals tested for possible autoimmune diseases were found to be positive for anti-Ro52 and/or anti-Ro60 autoantibodies. The autoantibodies were detected using a line immunoblot assay (EUROLINE: ANA Profile 3, EUROIMMUN, Lübeck, Germany). The method used provides a qualitative *in vitro* assay for fourteen autoantibodies to extractable cellular antigens: nRNP/Sm, Sm, Ro-60, Ro-52, La, Scl-70, PM-Scl, Jo-1, centromere protein B (CENP-B), PCNA, nucleosomes, histones, ribosomal P-proteins, and M2. Antinuclear antibodies (ANA) were evaluated by indirect immunofluorescence assay on HEp-2 cells (NOVA Lite Hep-2 ANA, Inova Diagnostics, Inc. San Diego CA, USA), while anti-dsDNA, were tested by ELISA (EUROIMMUN, Lübeck, Germany).

Clinical and laboratory data

From the 508 individuals with anti-Ro52 and/or anti-Ro60 positive sera, medical records of 272 patients were available for review. Demographic (age, gender), clinical (diagnosis at last follow-up visit, clinical manifestations and disease characteristics), and biological data (complete blood count, chemistry, hyper-gammaglobulinaemia, serum C3 and C4 complement levels, anti-β2 GPI-glycoproteins, anti-cardiolipins, rheumatoid factor, anti-cyclic citrullinated peptides, anti-thyroglobulin, anti-thyroid peroxidase) were recorded from the patients' medical files. Organ involvement (skin, mucosae, joints/muscles, thyroid, lung, heart, liver, gastrointestinal track, kidney, as well as peripheral and central nervous system) at disease onset and/or during follow-up was defined based on particular symptoms, signs, laboratory, radiologic and/or histopathologic data. Each patient's pre-existing diagnosis was re-evaluated at last follow-up visit to meet the most recent classification criteria for all autoimmune diseases present (18-25).

The study was approved by the Ethical/Scientific Committee of the Euroclinic Hospital of Athens (no. 111, 21/03/18) and the patients provided informed consent in accordance with the declaration of Helsinki.

Statistical analysis

Statistical analysis was performed using SPSS 21.0 (SPSS Inc) and significance was defined as $p<0.05$ throughout. Descriptive statistics were used with frequencies and percentages for qualitative variables and mean and

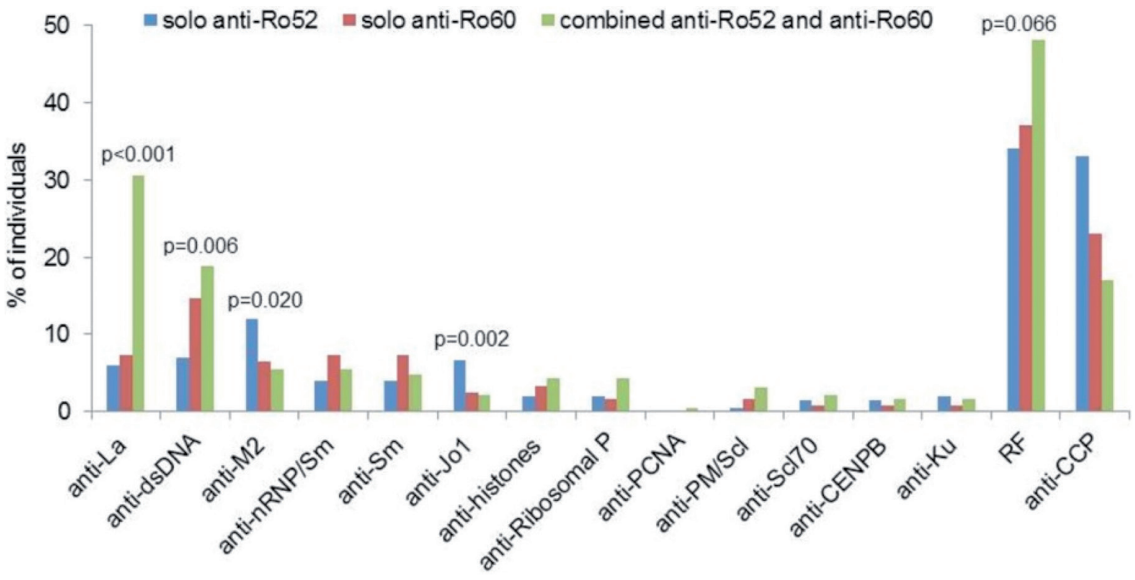

Fig. 2. Coexistence of other autoantibodies with anti-Ro52 and/or anti-Ro60 reactivities in the entire cohort (n=508).

standard deviation for quantitative variables. Associations of anti-Ro52 without anti-Ro60 (solo anti-Ro52), anti-Ro60 without anti-Ro52 (solo anti-Ro60) and combined anti-Ro52 and anti-Ro60 seropositivity with clinical and biological variables were analysed using chi square or Fisher's exact tests.

Results

Autoantibody profile

From the 508 individuals with positive anti-Ro52 and/or anti-Ro60 autoantibodies, 425 (84%) were women. Similarly, 241 (89%) of the 272 anti-Ro52 and/or anti-Ro60 positive patients with available medical records were women with a mean age of 48.0±16.1 years, mean disease duration of 9.6±8.5 years and a mean follow-up period of 6.8±5.5 years. The autoantibody profile of the 508 individuals' sera is shown in Figure 1a. Among the anti-Ro autoantibodies, the combination of anti-Ro52 and anti-Ro60 reactivity was the most frequent one followed by solo anti-Ro52, anti-La and solo anti-Ro60. The rest of the autoantibodies detected in these individuals' sera were primarily anti-cardiolipins, anti-β_2GPI, anti-dsDNA, anti-M2, anti-Sm, anti-nRNP/Sm and anti-Jo1 autoantibodies (Fig. 1A).

The serum autoantibody profile of the 272 patients with available medical records had a similar autoantibody distribution with that observed in the 508 individulas' sera (Fig. 1B). Among the 508 individuals, 86% had positive ANA, (titre ≥1:160) with a nuclear pattern in 75% and/or a cytoplasmic pattern in 25% of them. Similarly, 91% of the 272 patients had positive ANA with a nuclear pattern in 80% and/or a cytoplasmic pattern in 20% of them.

Associations of other autoantibodies with anti-Ro reactivity

Evaluation of the coexistence of other autoantibodies with anti-Ro52 and/or anti-Ro60 reactivity revealed that anti-Ro52 without anti-Ro60 was more frequently found either together with anti-M2 (p=0.020) or with anti-Jo1 (p=0.002) autoantibodies, while it showed a trend also to be found together with anti-cyclic citrullinated peptides (anti-CCP, p=0.077) (Fig. 2). Presence of solo anti-Ro60 autoantibodies had a tendency (not statistically significant) to be found together with anti-nRNP/Sm and anti-Sm (p=0.070) autoantibodies. The combination of anti-Ro52 and anti-Ro60 autoantibodies was observed to coexist significantly more often with anti-La (p<0.001) and/or anti-dsDNA (p=0.006), while it showed a tendency to coexist with rheumatoid factor (RF, p=0.066) (Fig. 2).

Disease diagnosis and anti-Ro reactivity

The diagnoses of the 272 patients with available medical records are shown in Table I. The most frequent diagnosis among these patients was SS (32%), followed by SLE (18%), undifferentiated connective tissue disease (UCTD, 14%), rheumatoid arthritis (RA, 10%), idiopathic inflammatory myopathy (IIM, 4%) and primary biliary cholangitis (PBC, 3%).

When stratifying patients based on their anti-Ro52 and/or anti-Ro60 seropositivity, two thirds of the SS patients and more than half of the SLE patients were anti-Ro52 and anti-Ro60 positive (Table I). The majority of patients with IIM (82%) and PBC (78%) had solo anti-Ro52 positivity. RA patients were more frequently found to possess solo anti-Ro52 (56%) followed by combined anti-Ro52 and anti-Ro60 (33%) reactivity and less frequently solo anti-Ro60 (11%). In the sera of patients which were characterised as having an UCTD, anti-Ro52 alone and combined anti-Ro52 and anti-Ro60 autoantibodies were similarly often present, while solo anti-Ro60 autoantibodies were less frequently detected (Table I).

Among SS patients, anti-centromere (ACA) reactivity was observed in four SS patients with solo anti-Ro52 and in three with anti-Ro52 and anti-Ro60 reactivity. All these SS patients manifested Raynaud's phenomenon, more than half (n=4) had oesophageal dysmotility and one had also pulmonary hypertension (from which he passed away). This subgroup of SS patients fulfills the clinical phenotype of ACA-positive SS patients which our group pointed out a long time ago (26, 27).

Among the SLE patients, almost all

with any anti-Ro52 and/or anti-Ro60 reactivity had arthralgias while sicca manifestations were very rare. Only 7/50 SLE patients had in their sera antibodies to Ro52 without Ro60 and four of those had also anti-cardiolipin antibodies. Of note, reactivity to anti-La autoantigens was observed in SLE patients' sera (7/50) only when anti-Ro60 as well as anti-dsDNA reactivity was present.

More than half of the 27 patients diagnosed as RA had solo anti-Ro52 reactivity in their sera while the rest had combined anti-Ro52 and anti-Ro60 reactivity except three with solo anti-Ro60 reactivity (Table I). These patients, in contrast to previous observations (28, 29), did not have sicca manifestations while 12/27 were positive for both RF and anti-CCP autoantibodies.

In eight out of eleven patients with IIM, a complete serological evaluation for myositis specific autoantibodies had been additionally performed. Half of the patients with IIM had clinical and/or serological manifestations of anti-synthetase syndrome, primarily with lung involvement. In two of them anti-Jo1 autoantibody was detected five and ten years respectively following IIM diagnosis, while in other two none of the anti-synthetase or other myositis specific autoantibodies were detected. In these patients however the ANA pattern was cytoplasmic connoting possible reactivity to non-identified autoantigens. It is worth noting that two of the patients with IIM had also anti-mitochondrial reactivity without liver enzyme abnormalities.

Patients with UCTDs (n=39) showed mostly solo anti-Ro52 (38%) and combined anti-Ro52 and anti-Ro60 (46%) reactivities. The predominant presenting clinical manifestations of UCTD patients were arthralgias (64%), Raynaud's phenomenon (26%) and dry eyes (33%). These patients were followed for a mean of 4.9±3.8 years and repeated autoantibody testing in 30% of them did not show significant changes except one patient in whom anti-Jo1 autoantibody was newly detected 5 years after the first evaluation.

Forty two (86% women, mean age 48.9±18.7 years) out of 272 patients had only anti-Ro52 seropositivity without any other autoantibody detected in their sera. These patients with a mean follow-up period of 4.9±3.7 years were diagnosed as having UCTD (24%), RA (18%), IIM (9%), SS (9%), autoimmune/inflammatory syndrome induced by adjuvants (ASIA, 6%) while 9% were categorised as healthy. These healthy individuals were outpatients referred for autoantibody screening by GPs or other specialties without probably an absolute indication for autoantibody screening. One of them had only livedo reticularis, 1 had only mild leukopenia and 2 of them had periodically mild arthralgias only. The remaining patients were evenly distributed in all other diagnoses.

Table I. The different disease diagnoses among the 272 patients with available medical records and their corresponding prevalence in the subgroups of solo anti-Ro52, solo anti-Ro60 and combined anti-Ro52 and anti-Ro60 reactivities.

Diagnosis at last follow-up visit (n)	anti-Ro52 (n=87) n, (%)	anti-Ro60 (n=49) n, (%)	anti-Ro52 and anti-Ro60 (n=136) n, (%)
SS (86)	18 (21)	11 (13)	57 (66)
SLE (50)	7 (14)	14 (28)	29 (58)
UCTD (39)	15 (38)	6 (15)	18 (46)
RA (27)	15 (56)	3 (11)	9 (33)
IIM (11)	9 (82)	-	2 (18)
PBC (9)	7 (78)	1 (11)	1 (11)
SCLE (8)	2 (25)	3 (37)	3 (37)
DLE (6)	-	2 (33)	4 (67)
Healthy (6)	3 (50)	1 (17)	2 (33)
SSc (5)	1 (20)	1 (20)	3 (60)
MCTD (4)	-	2 (50)	2 (50)
AIH (3)	1 (33)	-	2 (67)
APS (2)	-	2 (100)	-
ASIA (2)	2 (100)	-	-
Coeliac disease (2)	-	-	2 (100)
ITP (2)	2 (100)	-	-
Vasculitis (2)	2 (100)	-	-
Retroperitoneal fibrosis (1)	1 (100)	-	-
PM/Scl (1)	-	1 (100)	-
Autoimmune ovarian failure (1)	-	1 (100)	-
Autoimmune hypophysitis (1)	1 (100)	-	-
PsA (1)	-	1 (100)	-
IBD (1)	1 (100)	-	-
AS (1)	-	-	1 (100)
PMR (1)	-	-	1 (100)

UCTD: undifferentiated connective tissue disease; SLE: systemic lupus erythematosus; DLE: discoid lupus erythematosus; SCLE: subacute cutaneous lupus erythematosus; APS: antiphospholipid syndrome; SS: Sjögren's syndrome; RA: rheumatoid arthritis; SSc: systemic sclerosis; IIM: idiopathic inflammatory myopathy; PBC: primary biliary cholangitis ; AIH: autoimmune hepatitis; MCTD: mixed connective tissue disease; PM/Scl: polymyositis/scleroderma overlap; ASIA: autoimmune/inflammatory syndrome induced by adjuvants; PsA: psoriatic arthritis; IBD: inflammatory bowel disease; ITP: idiopathic thrombocytopenic purpura; AS: ankylosing spondylitis; PMR: polymyalgia rheumatica.

Anti-Ro reactivity and specific clinical manifestations

When evaluating the associations of specific clinical manifestations from different systems and organs (Supplementary Table S1) with anti-Ro52 and/or anti-Ro60 reactivity, it was found that anti-Ro52 without anti-Ro60 reactivity correlated with ILD and PBC (Table II). From the patients with ILD that had anti-Ro52 without anti-Ro60 (n=12), eight had IIM, two had an UCTD, one had SS, and one RA. The majority of them (10/12) were anti-Jo1-positive. All patients with solo anti-Ro52 reactivity and ILD had a lung imaging pattern of usual interstitial pneumonia or non-specific interstitial pneumonia, except one which had bronchiolitis obliterans with organising pneumonia. From the rest of the ILD patients (n=9), eight had SS and one RA. In the sera of these patients we found mostly combined anti-Ro52 and anti-Ro60 reactivity (56%), anti-La (55%) as well as RF (86%) positivity.

The majority (7/9) of PBC patients had solo anti-Ro52 positivity, one had solo anti-Ro60 reactivity and one had combined anti-Ro52 and anti-Ro60 (Table II). Six out of the seven patients with solo anti-Ro52 reactivity were also anti-M2 positive and all of them had cytoplasmic ANA pattern. Interestingly none of the PBC patients was anti-La positive, although half of them had sicca symptoms.

Anti-Ro60 reactivity without anti-Ro52 did only associate with oral ulcers (Table II), while the combination of anti-Ro52 and anti-Ro60 was significantly more prevalent in patients demonstrating salivary gland enlargement (SGE), interstitial kidney disease and sicca symptoms (Table II). All patients with interstitial kidney disease (n=6) had SS. When evaluating the concurrent positivity of anti-Ro52, anti-Ro60 and anti-La autoantibodies (n=52), this combination occurred significantly more frequently in patients with sicca symptoms (p=0.04), keratoconjunctivitis sicca (p=0.036), SGE (p<0.001), lymphoma (p=0.03), leukopenia (p=0.008), diffuse hypergammaglobulinaemia (p=0.003), RF positivity (p=0.005) and C4 hypocomplementaemia (p=0.052). The most frequent diagnosis in these patients was SS (73%), followed by SLE (19%), RA (4%), UCTD (2%) and SSc (2%).

Table II. Associations of clinical manifestations from all different systems and organs with anti-Ro52 and/or anti-Ro60 reactivity.

Clinical manifestations	solo anti-Ro52, n (%)	solo anti-Ro60, n (%)	combined anti-Ro52 and anti-Ro60, n (%)
ILD (n=21)	12 (57)*,**	1 (5)***	8 (38)
PBC (n=9)	7 (78)*,**	1 (11)	1 (11)
Oral ulcers (n=4)	0	3 (75)***	1 (25)
SGE (n=35)	9 (26)*	3 (8)	23 (66)**,***
Interstitial kidney disease (n=6)	0	0	6 (100)**,***
Sicca symptoms (n=110)	31 (28)	16 (14)	63 (57)**,***

ILD: interstitial lung disease; PBC: primary biliary cholangitis; SGE: salivary gland enlargement; sicca symptoms: comprising subjective xerostomia, subjective *xerophthalmia, ropy saliva and tongue atrophy.
*p<0.05 for the comparison between solo anti-Ro52 *vs*. solo anti-Ro60.
**p<0.05 for the comparison between solo anti-Ro52 *vs*. combined anti-Ro52 and anti-Ro60.
***p<0.05 for the comparison between solo anti-Ro60 *vs*. combined anti-Ro52 and anti-Ro60.

Sensitivity and specificity of anti-Ro reactivity for Sjögren's syndrome diagnosis

Anti-Ro positivity is used in the classification criteria for SS as an item having one of the highest weights (22). Thus, we opted to determine in our cohort the sensitivity and specificity of the isolated and the combined anti-Ro reactivity for SS diagnosis. Solo anti-Ro52 positivity had 21% sensitivity and 63% specificity, solo anti-Ro60 positivity had 13% sensitivity and 80% specificity, combined anti-Ro52 and anti-Ro60 positivity had 66% sensitivity and 57% specificity and anti-Ro52, anti-Ro60 and anti-La triple positivity had 43% sensitivity and 92% specificity for SS diagnosis.

Discussion

The present study identifies the clinical phenotype of consecutively identified, unselected individuals positive for anti-Ro52 and/or anti-Ro60 autoantibodies. Approximately half of the patients were diagnosed as having SS and SLE. It should be emphasised that the study includes outpatients as well as hospitalised patients investigated for renal, pulmonary, haematological or cardiac manifestations.

Similarly to other studies (12, 13, 30, 31) our work shows that among the sera tested, the combination of anti-Ro52 and anti-Ro60 autoreactivity was the most frequently observed followed by solo anti-Ro52 and less frequently by solo anti-Ro60 reactivity. The majority of our patients with the combination of anti-Ro52 and anti-Ro60 reactivity fulfilled classification criteria for SS or SLE as previously reported (2, 12) and vice versa, patients with SS and SLE exhibited in their majority a double reactivity against Ro52 and Ro60 autoantigens (13, 32, 33). Furthermore, as previously shown, patients with solo anti-Ro52 reactivity were diagnosed as having RA, IIM, PBC but also SS (16, 34). Solo anti-Ro60 reactivity was present more frequently in SLE patients (35).

In the sera of our patient population we confirmed the well-known associations between anti-Ro52 reactivity and anti-Jo-1 (13) and showed additionally that solo anti-Ro52 reactivity strongly associates to anti-M2 antibodies related or not to liver autoimmune injury targeting bile ducts (36). Anti-M2 antibodies are the hallmark of PBC (37), however, they can be also found in 7–12% of IIM patients without liver involvement (38).

Our study also confirmed the association between the combined anti-Ro52 and anti-Ro60 with anti-La and/or anti-dsDNA seropositivity (12, 31, 39, 40). Isolated anti-Ro60 seropositivity associated relatively more frequently with anti-Sm and nRNP/Sm reactivities (33).

From the results of our and other studies, several questions arise regarding the value and utility of the separate detection of anti-Ro52 and/or anti-Ro60 autoantibodies in clinical practice and more specifically in diagnosis/classification as well as in the pathogenetic mechanisms of autoimmune diseases.

The first one is if autoantibodies, like anti-Ro52 and/or anti-Ro60, observed in different diseases, can be included in the SS classification criteria and if their individual identification can aid disease diagnosis and prognosis.

The majority of our SS patients had in their sera both anti-Ro52 and anti-Ro60 reactivity. However, the sensitivity (66%) and specificity (57%) of this combined autoreactivity, as tested in this population with different clinical phenotypes, was relatively low. Yet, this dual specificity strongly correlated with SGE and interstitial kidney disease, manifestations of SS which are not included in the SS classification criteria (22). Because this correlation is not biased the relation of the dual autoantibody reactivity with SS becomes stronger.

The presence of solo anti-Ro60 reactiv-

ity revealed very low sensitivity (13%) but high specificity (80%) for SS. Of note, solo anti-Ro60 reactivity correlated strongly with oral ulcers, -characteristic manifestation of SLE-, and showed a tendency to co-exist with autoantibodies against Sm or nRNP/Sm, autoreactivity also found in SLE patients' sera. The correlation of solo anti-Ro60 with SLE was first advocated by Tan and his group in 1990 (35), while in parallel they described a strong correlation of solo anti-Ro52 reactivity with SS. Subsequent studies however did not substantiate this dichotomy, but it appears that both autoreactivities are present in the sera of SS and SLE patients (41) and in SLE they are frequently followed by autoreactivity to dsDNA and nRNP/Sm autoantigens (33).

It is of interest that while solo anti-Ro52 reactivity was low (21%) in SS patients, it was high in patients with IIM (82%) and PBC (78%). The calculation of sensitivity and specificity of solo anti-Ro52 reactivity for SS was low; 21% and 63% respectively. Indeed in our population-comprised of patients that had anti-Ro52 and/or anti-Ro60 positivity irrespectively of clinical symptoms and different diagnoses-, sensitivity and specificity of anti-Ro52 and anti-Ro60 autoantibodies for SS classification was low. Ro/SSA was historically considered a uniform antigen system. Nonetheless, now there is strong evidence that Ro52 and Ro60 antigens are not part of a stable macromolecular complex, but are located in different cellular compartments, have different functions, are encoded by different genes and are associated with different clinical phenotypes (6-10). A large number of commercially available kits and home-made techniques have been used to detect autoantibodies against Ro/SS-A, including double immunodiffusion (Ouchterlony), counter immunoelectrophoresis, Western Blot and ELISA. Nowadays, for several reasons (different nature of the antigens used in each assay; anti-Ro52 positive sera without anti-Ro60 and anti-La reactivity can be missed by classic anti-Ro/SSA detection methods) these techniques are slowly abandoned in everyday clinical practice and anti-Ro52 and anti-Ro60 tend to be identified by techniques that allow their separate detection during a serological examination. Bearing in mind all the above, although the current SS classification criteria consider the "global" anti-Ro/SSA reactivity, we suggest that a separate weight could be given to the solo reactivities and to the combined one.

The second question arising from the results of this study is if solo anti-Ro52 or combined anti-Ro52 with anti-Ro60 reactivity in SS defines different disease phenotypes and prognosis.

Previous studies from our group have divided SS patients in two categories according to clinical and serological findings present at disease diagnosis. In those with SGE, purpura and low serum C4 levels who have high risk for morbidity, lymphoma development and mortality (type I) and those without the above manifestations who have low risk for increased morbidity (type II) (42, 43). Our observations showed that the combination of antibodies to Ro52, Ro60 and La highly correlates, in addition to sicca manifestations – which are included in the SS diagnostic criteria – with SGE, lymphoma, leukopenia, diffuse hypergammaglobulinaemia, C4 hypocomplementaemia and RF positivity, all manifestations characteristic of the type I SS. SS patients with anti-Ro52 without anti-Ro60 had a milder clinical profile expressed by sicca manifestations and arthritis. One third of the SS patients with solo anti-Ro52 (6/18) had reactivity also to La autoantigen. Despite the presence of anti-La, these patients had a similar clinical profile with solo anti-Ro52 SS patients. All these patients have the characteristics of type II SS patients (44, 45). During their follow-up (mean of 5.0±4.1 years) their autoantibody profile did not change. These data suggest that the separate evaluation of the two anti-Ro antibodies in SS patients seems to be of great importance, in combination with other clinical and laboratory manifestations, for defining prognosis and instituting early therapeutic intervention.

Another question often posed by practicing physicians is: what is the significance of testing separately anti-Ro52 and anti-Ro60 autoantibodies, if the clinical phenotype does not fulfill criteria for any autoimmune disease? Such examples are individuals that demonstrate skin rashes or arthralgia or thrombocytopenia or even interstitial lung disease as sole manifestations. We showed that individuals with interstitial lung disease and solo anti-Ro52 antibodies did not fulfill criteria for SS but the presence of solo anti-Ro52 reactivity suggested that the lung injury has an autoimmune basis on the grounds of myositis related autoreactivity. The lung involvement related to SS or RA was characterised by the presence of combined anti-Ro52 and anti-Ro60 autoantibodies. Thus, our results suggest that in patients with ILD and solo anti-Ro52 in their sera, the underlying autoimmune disease is unlikely to be SS and further evaluation is needed for possible presence of myositis specific autoantibodies.

It is important to emphasise, however, that neither anti-Ro52 nor anti-Ro60 nor their combined presence in the sera of individuals with different autoimmune diseases is associated with any specific clinical manifestation. However, the presence of other autoantibodies, along with anti-Ro reactivity relates to specific clinical manifestations. For example, anti-synthetases strongly associate with ILD, ACA with Raynaud's phenomenon and pulmonary hypertension and anti-mitochondrial antibodies with autoimmune cholangitis. The co-occurrence of anti-Ro reactivity with other autoantibodies along with specific clinical manifestations may indicate common pathways triggering autoimmunogenicity in these disorders. We can thus suggest two pathways: one which is mainly responsible for the autoreactivity against synthetases, mitochondrial enzymes and anti-Ro52 and the other against La, DNA and spliceosomes accompanied with that to anti-Ro52 in combination to anti-Ro60.

Taking into account that La antigen plays a significant role in the cellular translation under stress (46, 47), that Ro60 antigen acts as quality-control for misfolded RNA (10), that Ro52 antigen acts in the process of ubiquitination (11), that synthetases are enzymes which attach the appropriate amino acid

onto its tRNA (48), that the mitochondrial enzymes which act as autoantigens are important for the maintenance for glucose homeostasis (49), and that spliceosomes remove introns from transcribed pre-mRNA (50), we could speculate that anti-Ro-related syndromes have as common mediator, a stressed cell which may play a role in the triggering and development of autoreactivity. The resulting clinical expression may have different forms according to the cause of stress and the affected targeted cells and tissues. Further delineation of the role of auto-antigens in the cellular function combined with meticulous clinical studies may support the effort for the delineation of the pathogenesis of autoimmune disorders.

Our work has strengths and weaknesses. The primary strengths of our study are the large number of sera tested for autoantibodies to cellular antigens, all performed in a single laboratory with the same methodology. Coupled to that, another strong point is the fact that for more than half of the sera tested, detailed patient medical records were available with a long follow-up period, which enabled us to offer a realistic representation of the diagnostic range associated with anti-Ro52 and/or anti-Ro60 autoreactivity. Individuals with this type of autoreactivity exhibit a wide clinical spectrum ranging from organ specific to systemic disorders and from subclinical pathological entities to well-defined syndromes.

On the other hand, the retrospective design of our study could be considered a weakness. In addition, another limitation, although not the scope of this work, lies within one of the strengths of our study, namely the use of one method for the detection of autoantibodies to cellular antigens.

This work however, can stimulate a setup of prospective multicentre studies in which a comparison of different methods commonly used to detect anti-Ro reactivity could provide valuable information on the sensitivity and specificity of the different methods, compare their results in terms of significant clinical value and thereby confirm or disprove the findings and conclusions of this work.

References

1. BIZZARO N: Autoantibodies as predictors of disease: the clinical and experimental evidence. *Autoimmun Rev* 2007; 6: 325-33.
2. FRANCESCHINI F, CAVAZZANA I: Anti-Ro/SSA And La/SSB Antibodies. *Autoimmunity* 2005; 38: 55-63.
3. ANDERSON JR, GRAY KG, BECK JS, BUCHANAN WW, McELHINNEY AJ: Precipitating auto-antibodies in the connective tissue diseases. *Ann Rheum Dis* 1962; 21: 360-9.
4. CLARK G, REICHLIN M, TOMASI TB JR: Characterization of a soluble cytoplasmic antigen reactive with sera from patients with systemic lupus erythematosus. *J Immunol* 1969; 102: 117-22.
5. ALSPAUGH MA, TAN EM: Antibodies to cellular antigens in Sjögren's Syndrome. *J Clin Invest* 1975; 55: 1067-73.
6. DEUTSCHER SL, HARLEY JB, KEENE JD: Molecular analysis of the 60-kDa human Ro ribonucleoprotein. *Proc Natl Acad Sci USA* 1988; 85: 9479-83.
7. BEN CHETRIT E, GANDY BJ, TAN EM, SULLVAN KF: Characterization, Isolation and SS-A/Ro, of a cDNA clone encoding the 60-kD component of the human Ribonucleoprotein Autoantigen. *J Clin Invest* 1989; 83: 1284-92.
8. CHAN EK, HAMEL JC, BUYON JP, TAN EM: Molecular definition and sequence motifs of the 52-kD component of human SS-A/Ro autoantigen. *J Clin Invest* 1991; 87: 68-76.
9. KONG HJ, ANDERSON DE, LEE CH *et al*.: Cutting edge: autoantigen Ro52 is an interferon inducible E3 ligase that ubiquitinates IRF-8 and enhances cytokine expression in macrophages. *J Immunol* 2007; 179: 26-30.
10. FUCHS G, STEIN AJ, FU C, REINISCH KM, WOLIN SL: Structural and biochemical basis for misfolded RNA recognition by the Ro autoantigen. *Nat Struct Mol Biol* 2006; 13: 1002-9.
11. DEFENDENTI C, ATZENI F, SPINA MF *et al*.: Autoimmunity Reviews Clinical and laboratory aspects of Ro/SSA-52 autoantibodies. *Autoimmun Rev* 2011; 10: 150-4.
12. PEENE I, MEHEUS L, VEYS EM, DE KEYSER F: Diagnostic associations in a large and consecutively identified population positive for anti-SSA and/or anti-SSB: the range of associated diseases differs according to the detailed serotype. *Ann Rheum Dis* 2002; 61: 1090-4.
13. MENÉNDEZ A, GÓMEZ J, ESCANLAR E, CAMINAL-MONTERO L, MOZO L: Clinical associations of anti-SSA/Ro60 and anti-Ro52/TRIM21 antibodies: Diagnostic utility of their separate detection. *Autoimmunity* 2013; 46: 32-9.
14. WODKOWSKI M, HUDSON M, PROUDMAN S *et al*.: 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. *Clin Exp Rheumatol* 2015; 33 (Suppl. 91): S131-5.
15. RETAMOZO S, AKASBI M, BRITO-ZERÓN P *et al*.: Anti-Ro52 antibody testing influences the classification and clinical characterisation of primary Sjögren's syndrome. *Clin Exp Rheumatol* 2012; 30: 686-92.
16. YAMASAKI Y, SATOH M, MIZUSHIMA M *et al*.: Clinical subsets associated with different anti- aminoacyl transfer RNA synthetase antibodies and their association with coexisting anti-Ro52 . *Mod Rheumatol* 2015; 26: 403-9.
17. FRITSCH C, HOEBEKE J, DALI H *et al*.: 52-kDa Ro/SSA epitopes preferentially recognized by antibodies from mothers of children with neonatal lupus and congenital heart block. A*rthritis Res Ther* 2006; 8: R4.
18. MOSCA M, NERI R, BOMBARDIERI S: Undifferentiated connective tissue diseases (UCTD): a review of the literature and a proposal for preliminary classification criteria. *Clin Exp Rheumatol* 1999; 17: 615-20.
19. ARINGER M, COSTENBADER K, DAIKH D *et al*.: 2019 European League Against Rheumatism/American College of Rheumatology classification criteria for systemic lupus erythematosus. *Ann Rheum Dis* 2019; 78: 1151-59.
20. LUNDBERG IE, TJÄRNLUND A, BOTTAI M *et al*.: 2017 European League Against Rheumatism/American College of Rheumatology classification criteria for adult and juvenile idiopathic inflammatory myopathies and their major subgroups. *Ann Rheum Dis* 2017; 76: 1955-64.
21. ALETAHA D, NEOGI T, SILMAN AJ *et al*.: 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum* 2010; 62: 2569-81.
22. SHIBOSKI CH, SHIBOSKI SC, SEROR R *et al*.: 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjögren's Syndrome: A Consensus and Data-Driven Methodology Involving Three International Patient Cohorts. *Arthritis Rheumatol* 2017; 69: 35-45.
23. VAN DEN HOOGEN F, KHANNA D, FRANSEN J *et al*.: 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. *Arthritis Rheum* 2013; 65: 2737-47.
24. TAAL BG, SCHALM SW, TEN KATE FW, HERMANS J, GEERTZEN RG, FELTKAMP BE: Clinical diagnosis of primary biliary cirrhosis: a classification based on major and minor criteria. *Hepatogastroenterology* 1983; 30: 178-82.
25. SHOENFELD Y, AGMON-LEVIN N: 'ASIA' autoimmune/inflammatory syndrome induced by adjuvants. *J Autoimmun* 2011; 36: 4-8.
26. VLACHOYIANNOPOULOS PG, DROSOS AA, WIIK A, MOUTSOPOULOS HM: Patients with anticentromere antibodies, clinical features, diagnoses and evolution. *Br J Rheumatol* 1993; 32: 297-301.
27. BOURNIA VK, DIAMANTI KD, VLACHOYIANNOPOULOS PG, MOUTSOPOULOS HM: Anticentromere antibody positive Sjögren's Syndrome: a retrospective descriptive analysis. *Arthritis Res Ther* 2010; 12: R47.
28. MOUTSOPOULOS HM, SKOPOULI FN, SARRAS AK *et al*.: Anti-Ro(SSA) positive rheumatoid arthritis (RA): a clinicoserological group of patients with high incidence of D-penicillamine side effects. *Ann Rheum Dis* 1985; 44: 215-9.
29. STEWART I, RIDWAY JC: Anti-Ro positive rheumatoid arthritis. *Ann Rheum Dis* 1986; 45: 86-7.

30. DUGAR M, COX S, LIMAYE V, GORDON TP, ROBERTS-THOMSON PJ: Diagnostic utility of anti-Ro52 detection in systemic autoimmunity. *Postgr Med J* 2010; 86: 79-82.
31. ROBBINS A, HENTZIEN M, TOQUET S *et al*.: Diagnostic utility of separate antibody detection in autoimmune diseases. *Front Immunol* 2019; 10: 444.
32. GÁL I, LAKOS G, ZEHER M: Comparison of the anti-Ro/SSA autoantibody profile between patients with primary and secondary Sjögren's syndrome. *Autoimmunity* 2000; 32: 89-92.
33. MENÉNDEZ A, GÓMEZ J, CAMINAL-MONTERO L, DÍAZ-LÓPEZ JB, CABEZAS-RODRÍGUEZ I, MOZO L: Common and specific associations of anti-SSA/Ro60 and anti-Ro52/TRIM21 antibodies in systemic lupus erythematosus. *ScientificWorldJournal* 2013; 2013: 832789.
34. GHILLANI P, ANDRÉ C, TOLY C *et al*.: Autoimmunity reviews clinical significance of anti-Ro52 (TRIM21) antibodies non-associated with anti-SSA 60 kDa antibodies: results of a multicentric study. *Autoimmun Rev* 2011; 10: 509-13.
35. BEN-CHETRIT E, FOX RI, TAN EM: Dissociation of immune responses to the SS-A (Ro) 52-kd and 60-kd polypeptides in systemic lupus erythematosus and Sjögren's syndrome. *Arthritis Rheum* 1990; 33: 349-55.
36. GRANITO A, MURATORI P, MURATORI L *et al*.: Antibodies to SS-A/Ro-52kD and centromere in autoimmune liver disease: a clue to diagnosis and prognosis of primary biliary cirrhosis. *Aliment Pharmacol Ther* 2007; 26: 831-8.
37. LLEO A, MARZORATI S, ANAYA JM, GERSHWIN ME: Primary biliary cholangitis: a comprehensive overview. *Hepatol Int* 2017; 11: 485-99.
38. MAEDA MH, TSUJI S, SHIMIZU J: Inflammatory myopathies associated with anti-mitochondrial antibodies. *Brain* 2012; 135: 1767-77.
39. MANOUSSAKIS MN, TZIOUFAS AG, PANGE PJ, MOUTSOPOULOS HM: Serological profiles in subgroups of patients with Sjögren's syndrome. *Scand J Rheumatol Suppl* 1986; 61: 89-92.
40. HARLEY JB, ALEXANDER EL, BIAS WB *et al*.: Anti-Ro (SS-A) and anti-La (SS-B) in patients with Sjögren's syndrome. *Arthritis Rheum* 1986; 29: 196-206.
41. SLOBBE RL, PRUIJN GJ, DAMEN WG, VAN DER KEMP JW, VAN VENROOIJ WJ: Detection and occurrence of the 60- and 52-kD Ro (SS-A) antigens and of autoantibodies against these proteins. *Clin Exp Immunol* 1991; 86: 99-105.
42. SKOPOULI FN, DAFNI U, IOANNIDIS JP, MOUTSOPOULOS HM: Clinical evolution, and morbidity and mortality of primary Sjögren's syndrome. *Semin Arthritis Rheum* 2000; 29: 296-304.
43. FRAGKIOUDAKI S, MAVRAGANI CP, MOUTSOPOULOS HM: Predicting the risk for lymphoma development in Sjogren syndrome. *Medicine* (Baltimore) 2016; 95: e3766.
44. IOANNIDIS JP, VASSILIOU VA, MOUTSOPOULOS HM: Long-term risk of mortality and lymphoproliferative disease and predictive classification of primary Sjögren's syndrome. *Arthritis Rheum* 2002; 46: 741-7.
45. MOUTSOPOULOS HM: Sjögren's syndrome: A forty-year scientific journey. *J Autoimmun* 2014; 51: 1-9.
46. KIM YK, JANG SK: Continuous heat shock enhances translational initiation directed by internal ribosomal entry site. *Biochem Biophys Res Commun* 2002; 297: 224-31.
47. VINAYAK J, MARRELLA SA, HUSSAIN RH, ROZENFELD L, SOLOMON K, BAYFIELD MA: Human La binds mRNAs through contacts to the poly(A) tail. *Nucleic Acids Res* 2018; 46: 4228-40.
48. MIRANDE M: The Aminoacyl-tRNA Synthetase Complex. *Subcell Biochem* 2017; 83: 505-22.
49. PATEL MS, NEMERIA NS, FUREY W, JORDAN F: The pyruvate dehydrogenase complexes: structure-based function and regulation. *J Biol Chem* 2014; 289: 16615-23.
50. WILL CL, LÜHRMANN R: Spliceosome structure and function. *Cold Spring Harb Perspect Biol* 2011; 3: pii: a003707.