The value of separate detection of anti-Ro52, anti-Ro60 and anti-SSB/La reactivities in relation to diagnosis and phenotypes in primary Sjögren's syndrome

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Abstract

Objective
Autoantibody detection is an essential step in pSS diagnosis. However, the value of separate anti-Ro52, anti-Ro60 and anti-SSB/La detection in pSS diagnosis and phenotyping has not been extensively studied. This study aimed to explore disease characteristics of anti-SSA/Ro positive, suspected and definite pSS patients, in relation to serological profiles based on anti-Ro52, anti-Ro60 and anti-SSB/La reactivity.

Methods
Of 187 anti-SSA/Ro positive patients included in the Belgian Sjögren's Syndrome Transition Trial (BeSSTT), 155 were considered definite pSS patients, due to fulfilment of the 2016 ACR-EULAR classification criteria, and 32 suspected, due to reactivity against SSA/Ro without presence of other criteria. None of the patients met any of the ACR-EULAR exclusion criteria for pSS. Patients were grouped based on the presence of anti-Ro52, anti-Ro60 and anti-SSB/La antibodies.

Results
Mono-reactivity against Ro60 or Ro52, double reactivity against Ro52/Ro60 and triple reactivity against Ro52/Ro60 and SSB was detected in respectively 30, 23, 70 and 60 patients. Mono-anti-Ro60 positive patients showed the least pSS features. Mono-anti-Ro52 positive patients reported a significantly higher dryness burden (p=0.016) and tended toward more salivary gland ultrasound (SGUS) abnormalities (p=0.054) than mono-anti-Ro60 positives. Double positive patients showed similar characteristics as mono-anti-Ro52 positive patients, whereas triple positive patients showed lowest unstimulated salivary flow rates (p=0.002) and Schirmer tests (p=0.002), highest ocular staining scores (p<0.001), most positive labial salivary gland biopsies (p=0.039), most laboratory abnormalities compatible with B-cell hyperactivity and highest SGUS scores (p<0.001) compared to other patient groups.

Conclusion
These data indicate that separate detection of anti-Ro52, anti-Ro60 and anti-SSB/La reactivity is not only relevant towards pSS diagnosis, but markedly aids in patient stratification and evaluation of disease burden. Our results suggest a stepwise model in which mono-reactivity against Ro60 displayed the least objective and subjective glandular pSS features, whereas glandular abnormalities and signs of B-cell hyperactivity were most present in patients showing triple reactivity against Ro60, Ro52 and SSB/La.

Key words
autoimmune diseases, Sjögren's syndrome, autoantibodies, phenotype, diagnosis

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Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease typically manifesting as oral and ocular dryness, often associated with fatigue and diffuse arthralgia, mainly affecting middle-aged women (1). Objective sicca has a central place in the 2016 ACR/EULAR classification criteria. In addition, pSS classification requires evidence of autoimmunity either by the presence of anti-SSA/Ro autoantibodies or a positive labial salivary gland biopsy defined by a focus score of at least one (2). Besides the hallmark of anti-SSA/Ro antibodies, B-cell hyperactivity is also exemplified by anti-SSB/La antibodies, elevated serum IgG and rheumatoid factor (RF), and an increased risk of B-cell lymphoma (3).

Anti-SSA/Ro autoantibodies include reactivity towards two components, Ro60 and Ro52 (4). Depending on the criteria used, 33-74% of pSS patients manifest reactivity against the SSA/Ro antigen, 55–70% against the Ro52 and 45–70% against the Ro60 antigen (5, 6). About two thirds of patients demonstrate double reactivity against both antigens (7, 8). Anti-SSB/La reactivity is detected in 58% of anti-SSA/Ro positive pSS patients (9). However, classic anti-SSA/Ro detection methods preferentially detected anti-Ro60 antibodies and failed to detect mono-anti-Ro52 positive sera (10). More recently, several authors have confirmed the relevance of anti-Ro52 reactivity and supported routine independent detection of anti-Ro60 and anti-Ro52 antibodies (8, 11, 12). Reactivity against anti-SSB/La is no longer a classification criterion, as reactivity in the absence of anti-SSA/Ro antibodies was found not to be associated with key phenotypic features of pSS (13).

pSS is highly heterogenous, both in pathophysiology and presentation, which complicates the definition of homogeneous subgroups for clinical trials. The exact value of individual detection of anti-Ro52, anti-Ro60 and anti-SSB/La antibodies in relation to pSS diagnosis and disease burden has not been extensively studied.

This study aimed to explore disease characteristics of anti-SSA/Ro positive patients with suspected or definite pSS in relation to serological profiles, based on anti-Ro52, anti-Ro60 and anti-SSB/La reactivity.

Methods

Study design and participants

The Belgian Sjögren's Syndrome Transition Trial (BeSSTT) is an observational prospective longitudinal cohort of patients with definite pSS, defined by fulfilment of the 2016 ACR/EULAR classification criteria, and of patients suspected of pSS, due to presence of at least one criterion, either objective sicca or presence of an immunological criterion. Patients are seen yearly for data collection. For these analyses only patients who exhibited mono-anti-Ro52, mono-anti-Ro60 or combined anti-Ro52/Ro60 reactivity, independently of the presence of anti-SSB/La antibodies, were included. Patients who met the 2016 ACR-EULAR exclusion criteria for pSS were removed from the analyses. Medical history was retrieved from the medical record, including past pSS disease manifestations such as B-cell lymphoma. Demographic and clinical data, extensive laboratory parameters, as well as measures quantifying ocular and oral dryness were collected. Ocular staining score (OSS) was assessed by an ophthalmologist using the Sjögren's International Collaborative Clinical Alliance (SICCA) scheme (15). Schirmer's test and unstimulated salivary flow rate (USFR) were determined according to the guidelines of the ACR/EULAR classification criteria (2). OSS was reported as the sum score of both eyes and Schirmer's test as the mean. Ultrasonographic evaluation of the salivary glands was performed alternately by two dedicated radiologists. Abnormalities were scored in real-time using the Hocevar score, a 0–48 scoring system with higher scores indicating more severe abnormalities. A score of at least 17 was considered positive (16). Results of labial salivary gland biopsies were recorded when available and focus score was determined (17). Patients completed the EULAR Sjogren's Syndrome Patient Reported Index (ESSPRI) (14). All data, except for the salivary gland biopsy, were

Table I. Clinical and laboratory parameters amongst serology-based patient groups.

	Mono-anti-Ro60 (n=30)	Mono-anti-Ro52 (n=23)	Double anti-Ro52/Ro60 (n=70)	Triple anti-Ro52/ Ro60 & SSB/La (n=60)	p
Age, mean (±SD)	49.3 (±14.0)	57.3 (±12.2)	51.1 (±15.8)	49.9 (±15.3)	0.177
Female gender, n (%)	26 (86.7)	21 (91.3)	62 (88.6)	54 (90.0)	0.944
ESSPRI, mean (±SD)	4.7 (±2.6)	6.3 (±2.0)	5.0 (±2.3)	5.6 (±2.2)	0.090
Pain	5.0 (±2.9)	5.7 (±2.5)	4.3 (±3.1)	4.3 (±3.1)	0.179
Dryness	3.6 (±3.0)	6.4 (±2.7)	5.0 (±3.4)	6.3 (±2.8)	0.001
Fatigue	5.5 (±3.1)	6.9 (±2.3)	5.6 (±2.7)	6.1 (±2.6)	0.235
Dryness symptoms, n (%)	18 (60.0)	22 (95.7)	55 (78.6)	55 (91.7)	<0.001
Age of onset sicca symptoms, mean (±SD)	42.3 (±13.1)	48.0 (±14.7)	41.5 (±15.9)	40.7 (±15.8)	0.315
Complaints of salivary gland swelling ever, n (%)	8 (26.7)	8 (34.8)	20 (28.6)	25 (41.7)	0.360
USFR (ml/5min), mean (±SD)	1.4 (±1.2)	0.8 (±0.9)	1.1 (±1.1)	0.7 (±0.8)	0.002
USFR ≤0.5ml/5min, n (%)	8 (26.7)	13 (56.5)	24 (34.3)	35 (58.3)	0.005
Schirmer (mm/5min), mean (±SD)	13.7 (±11.0)	8.4 (±8.5)	8.3 (±9.7)	5.4 (±6.3)	0.002
Schirmer ≤5 mm/5min, n (%)	10 (33.3)	15 (65.2)	51 (72.9)	48 (80.0)	<0.001
OSS, median (IQR)	2 (0-3)	3 (1-5)	3 (1-7)	5 (2-12)	<0.001
OSS ≥5, n (%)	3 (10.0)	3 (13.0)	13 (18.6)	21 (35.0)	0.013
LSGB: FS ≥1, n/n tot‡ (%)	2/13 (15.4)	4/8 (50.0)	16/28 (57.1)	11/17 (64.7)	0.039
pSS classification, n (%)	18 (60.0)	18 (78.3)	59 (84.3)	56 (93.3)	0.001
Hocevar, median (IQR)	8 (4-15)	17 (10-30)	19 (8-34)	33 (24-42)	<0.001
Hocevar ≥17, n (%)	7 (23.3)	12 (52.2)	37 (52.9)	54 (90.0)	<0.001
Lymphocytes (/µl), mean (±SD)	1808 (±753)	1508 (±544)	1344 (±695)	1357 (±566)	0.004
Lymphocytes <1000 (/µl), n (%)	3 (10.0)	5 (21.7)	20 (28.6)	16 (26.7)	0.207
β2-microglobulin* (mg/l, mean (±SD)	1.9 (±0.4)	2.2 (±0.5)	2.4 (±1.0)	2.5 (±0.9)	0.005
β2-microglobulin >2.53 mg/L*, n (%)	2 (6.7)	5 (21.7)	17 (24.3)	21 (35.0)	0.037
IgG (g/l), mean (±SD)	12.3 (±3.0)	12.0 (±4.1)	15.2 (±6.3)	17.1 (±5.0)	<0.001
IgG >16.0 g/l, n (%)	3 (10.0)	3 (13.0)	23 (32.9)	33 (55.0)	<0.001
RF (U/ml), mean (±SD)	11.3 (±0.1)	54.6 (±174.9)	53.8 (±107.5)	44.1 (±62.6)	<0.001
RF >40 (U/ml), n (%)	0 (0.0)	2 (8.7%)	16 (22.9)	17 (28.3)	0.006
C3 g/L, mean (±SD)	1.19 (±0.20)	1.25 (±0.22)	1.15 (±0.23)	1.16 (±0.29)	0.292
C3 <0.9 g/L, n (%)	1 (3.3)	1 (4.3)	6 (8.6)	11 (18.3)	0.115
C4 g/L, mean (±SD)	0.24 (±0.06)	0.21 (±0.07)	0.21 (±0.07)	0.19 (±0.06)	0.008
C4 <0.10 g/L, n (%)	0 (0.0)	1 (4.3)	3 (4.3)	2 (3.3)	0.779
ANA positivity, n (%)	29 (96.7)	22 (95.7)	70 (100.0)	59 (98.3)	0.228
ESSDAI, median (IQR)	1 (0-3)	2 (1-4)	2 (0-4)	2 (1-4)	0.326
Lymphoma history, n (%)	1 (3.3)	1 (4.3)	2 (2.9)	2 (3.3)	-

p-values based on one-way ANOVA, Kruskal-Wallis, Chi-squared or Fisher's exact tests as appropriate. Bonferroni correction was applied.
‡Reported as ratio of n with FS ≥1 on total number of available labial salivary gland biopsies. *Results missing in 15 patients.
ESSPRI: EULAR Sjögren's Syndrome Patient Reported Index; USFR: unstimulated salivary flow rate; OSS: ocular staining score; LSGB: labial salivary gland biopsy; FS: focus score; ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index.

obtained at the baseline visit in the BeSSTT clinical carepath between October 2019 and February 2022. The severity of the clinical disease manifestations was assessed using the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) (18).

The Ghent University Hospital ethics committee granted approval for this research project (BeSSTT: EC 2019/04542) and all participants provided written informed consent.

Autoantibody detection
Indirect immunofluorescence (IIF) on HEp-2000 cells was performed on all samples with description of the fluorescence intensity (1+ to 5+) and the antinuclear antibody fluorescence pattern. Subsequently independent of IIF, an enzyme-labelled anti-isotype assay (EliA) symphony (Thermo Fisher Scientific, REF:14-5671-01) was performed on all samples allowing the sensitive screening of a mixture of nu-

clear and cytoplasmic antigens. Samples that tested positive on the EliA symphony were further investigated using a more specific anti-ENA line blot (Euroimmun, REF:1590-1601-5 G), where among others anti-Ro52, anti-Ro60, anti-SSB/La, anti-Sm, anti-Cenp-B, anti-Jo-1 and anti-Scl-70 antibodies could separately be detected. Due to lower sensitivity of the anti-ENA blot for anti-Ro60 antibodies, this reactivity was confirmed using a
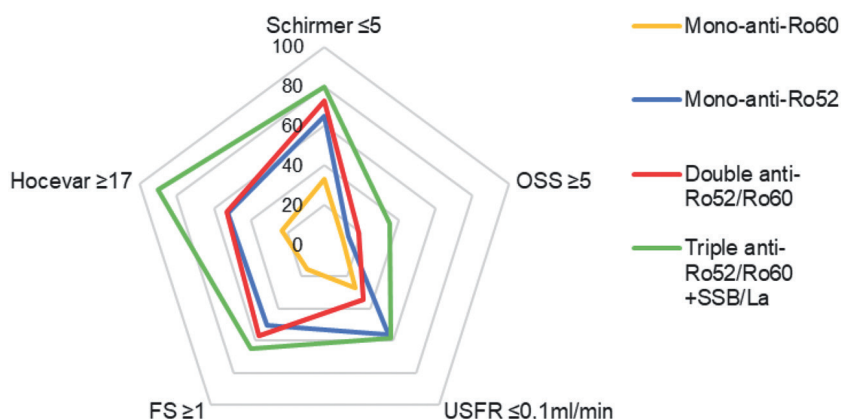
Fig. 1. Radar chart illustrating the prevalence of objective glandular features related to pSS key amongst serological profiles.
Radar chart illustrating the prevalence in percentage of objective glandular features related to pSS amongst patient groups defined by presence of anti-Ro60, anti-Ro52 and anti-SSB/La antibodies.
OSS: ocular staining score; USFR: unstimulated salivary flow rate; FS: focus score.

fluorescence enzyme immunoassay (Thermo Fisher Scientific, REF:14-5525-01) in case of a negative result on the line blot.

Statistical analysis
Study data were collected and managed using REDCap electronic data capture tools hosted at the Ghent University Hospital (19, 20). Statistical analyses were performed using SPSS (IBM Corp. Released 2021. IBM SPSS Statistics for Windows, v. 28.0. Armonk, NY: IBM Corp).
Differences between serology-based patient groups were determined using one-way ANOVA, Kruskal-Wallis, Chi-squared or Fisher's Exact tests as appropriate. Bonferroni correction was applied for post-hoc analyses to detect differences between individual serology-based subgroups. Binary logistic regression models were applied, with serology-based groups (mono-anti-Ro52, mono-anti-Ro60, double anti-Ro52/Ro60, triple anti-Ro52/Ro60 + anti-SSB/La) as independent variable. *p*-values ≤0.05 were considered statistically significant.

Results
Data of 187 anti-SSA/Ro positive patients were analysed. Mono-reactivity against Ro60 and Ro52 was detected in respectively 30 (16.0%) and 23 (12.3%) patients. Antibodies against both Ro52 and Ro60 were detected in 130 (69.5%) patients and anti-SSB/La antibodies

coincided in 60 (46.2%). These groups will further be referred to as mono-anti-Ro52, mono-anti-Ro60, double and triple positive. Due to low incidence of anti-SSB/La antibodies in the presence of only anti-Ro60 or anti-Ro52 antibodies (respectively 3 and 1 patients), these data were left out of the analyses.

Demographics and sicca in relation to serology-based groups
Patients were predominantly female with a mean ± SD age of 51.2±15.0 years. The proportion of patients reporting dryness and the overall dryness burden were lower in the mono-anti-Ro60 positive than in the mono-anti-Ro52 and triple positive groups (*p*<0.001 and *p*=0.001), whereas age of complaint onset was comparable between all serology-based groups (*p*=0.315). In line with these findings, less patients had aberrant unstimulated salivary flow rates (USFR) (*p*=0.005), positive Schirmer tests (*p*=0.001) and ocular staining scores (OSS) (*p*=0.013) in the mono-anti-Ro60 compared to the triple positive group. The proportion of positive Schirmer's tests was even significantly lower than in double and triple positive patient groups. Consequently, a lower proportion of mono-anti-Ro60 positive patients compared to double and triple positive patients was classified as pSS (*p*=0.001). No difference in systemic disease activity as assessed by ESSDAI was detected between serology-based groups (*p*=0.326), nor was there any

difference regarding history of synovitis, cutaneous manifestations, interstitial lung disease or neurologic complications. History of lymphoma was overall low and all affected patients fulfilled the pSS ACR/EULAR classification criteria (Table I).

Salivary gland involvement in relation to serology-based groups
Proportionally less mono-anti-Ro60 than triple positive patients showed a focus score of at least one on labial salivary gland biopsy (*p*=0.039). Salivary gland ultrasound (SGUS), assessed by Hocevar score, showed less abnormalities in the mono-anti-Ro60 positive versus the mono-anti-Ro52 and double positive patients, but this was only significant between the mono-anti-Ro60 and the double positive group (*p*=0.054 and *p*=0.002). Additionally, Hocevar scores in the triple positive were significantly higher than in all other patient groups (*p*<0.001).

Laboratory results in relation to serology-based groups
Double and triple positive patients showed most laboratory abnormalities with lower lymphocyte counts (*p*=0.004) and C4 levels (*p*=0.008), and higher serum β2-microglobulin (*p*=0.005), IgG (*p*<0.001) and RF (*p*<0.001) levels. Lymphocyte count and β2-microglobulin level did not significantly differ from the mono-anti-Ro52 positive group, IgG levels only significantly differed between the triple positive versus the other patient groups, and C4 was only significantly lower in the triple positive than in the mono-Ro60 positive patient group. Figure 1 illustrates the prevalence of objective glandular pSS features in the BeSSTT cohort in relation to serology-based patient groups. When solely looking at the 151 patients fulfilling the ACR/EULAR classification criteria, the conclusions described above remained unchanged.

Odds for pSS classification and pSS characteristics in relation to serology-based groups
Binomial logistic regression models with serology-based groups as independent variable showed that mono-

Table II. Association of serology-based group and phenotypic pSS features.

Phenotypic pSS features	Mono-anti-Ro52 vs. Mono-anti-Ro60		Double anti-Ro52/Ro60 vs. Mono-anti-Ro52		Triple anti-Ro52/Ro60 + anti-SSB/La vs. Double anti-Ro52/Ro60	
	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
Salivary gland swelling	1.5 (0.5-4.8)	0.524	0.8 (0.3-2.0)	0.574	1.8 (0.9-3.7)	0.119
Ocular sicca symptoms	9.2 (1.8-46.3)	0.007	0.2 (0.0-1.0)	0.045	1.7 (0.7-3.7)	0.213
Schirmer =<5	3.6 (1.1 -11.3)	0.030	1.4 (0.5-3.9)	0.485	1.6 (0.7-3.8)	0.257
OSS >=5	1.4 (0.2-7.4)	0.730	1.5 (0.4-6.0)	0.528	2.4 (1.1-5.5)	0.030
Oral sicca symptoms	12.0 (2.4-60.5)	0.003	0.2 (0.0-1.0)	0.045	2.6 (1.1-6.2)	0.032
USFR =<0.1ml/min	5.6 (1.1-11.3)	0.031	0.4 (0.2-1.0)	0.063	2.7 (1.3-5.5)	0.007
LSGB: FS >=1	5.5 (0.7-42.6)	0.103	1.3 (0.3-6.4)	0.720	1.4 (0.4-4.8)	0.616
Hocevar >=17	3.6 (1.1-11.6)	0.034	1.0 (0.4-2.6)	0.955	8.0 (3.1-21.1)	<0.001
pSS classification	2.4 (0.7-8.2)	0.163	1.5 (0.5-4.9)	0.508	2.6 (0.8-8.7)	0.118
Lymphocytes <1000 /mm³	2.5 (0.5-11.8)	0.247	1.5 (0.5-4.6)	0.478	0.9 (0.4-1.9)	0.730
β2-microglobulin >2.53 mg/L	4.2 (0.7 -24.2)	0.112	1.1 (0.3 -3.4)	0.918	1.7 (0.8 -3.7)	0.182
IgG >16.0 g/l	1.4 (0.2-7.4)	0.730	3.3 (0.9-12.1)	0.077	2.5 (1.2-5.1)	0.012
RF >40.0 U/mL	-	-	3.1 (0.7-14.7)	0.152	1.3 (0.6-2.9)	1.334
C3 <0.9 g/L	1.3 (0.1-22.3)	0.848	2.1 (0.2-18.1)	0.514	2.4 (0.8-6.9)	0.107
C4 <0.10 g/L	-	-	1.0 (0.1-10.0)	0.990	0.8 (0.1-4.8)	0.779

Stepwise change of odds ratios (OR's) of key pSS features between different serological profiles based on presence of anti-Ro60, anti-Ro52 and anti-SSB antibodies. OR's and 95% CI estimated using binary logistic regression models with serological profile as independent variable and phenotypic pSS features as dependent variables.
OSS: ocular staining score; USFR: unstimulated salivary flow rate; LSGB: labial salivary gland biopsy; FS: focus score.

anti-Ro60 positive patients had overall lowest odds to present with pSS-related abnormalities. Mono-anti-Ro52 positive patients were more likely to experience oral (p=0.003) and ocular dryness symptoms (p=0.007), and presented with more aberrant Schirmer tests (p=0.030), USFR's (p=0.031) and SGUS scores (p=0.034) compared to mono-anti-Ro60 positive. Double positive patients did not present significantly different than mono-anti-Ro52 positive patients albeit that mono-anti-Ro52 were slightly more likely to report sicca symptoms (p=0.045). Triple positive patients, however, showed significantly higher odds to experience oral dryness symptoms (p=0.032) and to present with an aberrant OSS (p=0.030), USFR (p=0.007) and SGUS (p<0.001) compared to double positive patients. These patients also had higher odds for aberrant IgG levels (p=0.012) compared to double positive patients.

Table II demonstrates the stepwise change of odds to present with an aberrant result for one of the pSS key features between serology-based groups.

Discussion

In this study, anti-SSA/Ro positive patients with suspected or definite pSS were stratified based on the presence of anti-Ro52, anti-Ro60 and anti-SSB/La antibodies. Clinical, ultrasonographic as well as laboratory parameters proved to be significantly associated with these serology-based groups, with mono-anti-Ro60 positive patients presenting with the least, and patients with triple anti-Ro60, anti-Ro52 and anti-SSB/La reactivity with the most abnormalities. pSS is characterised by the presence of autoantibodies, most typically against SSA/Ro and SSB/La. As seronegative patients may represent a different disease pathophysiology, we chose not to include these patients in this manuscript (21). Anti-SSA/Ro reactivity refers to two distinct autoantibodies, anti-Ro52 and anti-Ro60, which frequently coincide and are immunologically-linked, albeit that evidence about a physical link is lacking (6). Researchers of our group reported that the classical anti-SSA/Ro serological assays failed to detect anti-Ro52 antibodies, especially in the absence of anti-Ro60 antibodies (10). More recently, it was shown that anti-Ro52 antibodies were strongly associated with pSS features and other research suggested that separate detection of anti-Ro52 and anti-Ro60 antibodies adds value in the diagnosis of both pSS and other autoimmune diseases (11, 12, 22). However, to date there is no consensus on which assays to use for anti-SSA/Ro detection and furthermore

it is not always clear whether publications reporting anti-SSA/Ro reactivity even capture mono-anti-Ro52 antibodies. The 2016 ACR/EULAR classification criteria state that further validation studies are needed to support monospecific antibody assays for classification purposes (2). In our cohort, 78% of the patients with mono-anti-Ro52 reactivity were classified as pSS with most exhibiting other pSS features in addition to objective dryness. This clearly suggests the use of assays containing both antigens. As anti-Ro52 can be negative or not displaying any reliable nuclear or cytoplasmic staining on HEp-2 cells on indirect immunofluorescence, this pSS subgroup may be missed by primary care physicians since antinuclear antibody testing is the first test available in non-specialised standard healthcare settings (12, 23). Ideally, a predefined laboratory work-up for patients suspected with pSS should be available containing these three autoantigens.

It was previously shown that pSS patients with both anti-Ro52 and anti-Ro60 antibodies showed more B-cell hyperactivity and glandular inflammation than those with mono-anti-SSA/Ro reactivities. However, until now studies have never stratified pSS patients based on mono, double and triple reactivity against Ro52, Ro60 and SSB/La.(24)

While isolated anti-SSB/La reactivity is no longer retained as a pSS classification criterion as it appeared to be unrelated to key pSS features (13), the Big Data Project found most glandular disease activity in the pSS subgroup with isolated presence of anti-SSB/La antibodies. This study also showed a higher frequency of abnormal diagnostic tests and most systemic disease activity in the combined anti-SSA/Ro and anti-SSB/La positive, compared to patients with only anti-SSA/Ro or anti-SSB/La reactivity (9). Additionally, other authors stated that patients with a positive labial salivary gland biopsy and both anti-SSA/Ro and anti-SSB/La antibodies were younger at diagnosis, had longer disease duration and higher prevalence of lymphoma and hypergammaglobulinaemia compared to patients with solely anti-SSA/Ro antibodies (5). These data indicate a higher disease burden which is consistent with our findings, except that simultaneous, individual anti-Ro52, anti-Ro60 and anti-SSB detection was not accounted for in these studies.

Our results show a more profound involvement in patients with triple compared to double reactivity as illustrated by more pronounced dryness, both objective and subjective, markedly higher SGUS scores and higher IgG levels, while no differences were observed between mono-anti-Ro52 and double anti-SSA/Ro positive patients other than a higher RF level. The least clear pSS disease features were observed in the mono-anti-Ro60 positive group. This finding is supported by data of Armağan and colleagues that show numerically higher proportions of mono-anti-Ro52 positive patients presenting with aberrant Schirmer, OSS, USFR and labial salivary gland biopsy than of those with mono-anti-Ro60 antibodies (24). We believe our findings confirm and underscore the relevance of mono-anti-Ro52 in pSS (8, 11, 12) and challenge the conclusion of Veenbergen *et al*. stating that testing for isolated anti-Ro52 antibodies is of limited clinical value in SS (25). Moreover, for the first time an escalating effect of anti-Ro60, anti-Ro52 and anti-SSB/La presence amongst suspected pSS patients is suggested. Mono-anti-Ro60 positive patients present with the least clear phenotype, followed by the anti-Ro52 and double anti-SSA/Ro positive, and the triple positive patients presenting with most subjective and objective glandular disease expression and B-cell hyperactivity.

To date, no disease-modifying antirheumatic drugs have been licensed for patients with pSS, mainly because of the high heterogeneity in clinical presentation, complicating both the inclusion criteria and definition of reliable outcome measures for clinical trials. The selection of patients and primary study endpoints is crucial in this. Our data may allow for homogeneous patient stratification based on the serology profile and potentially outperform the stratification based on ESSDAI as the latter is often the limiting factor in patient recruitment. In case of treatment response, most triple positive patients for example, will be responders on the recently developed CRESS (26) and STAR (27) composite indexes since the majority of this subgroup has high ESSPRI, high Hocevar, high objective and subjective features of sicca and signs of B-cell hyperactivity.

Current understanding of the pathophysiological role of these autoantibodies and why exactly they usually co-occur is still an enigma. Despite extensive research on Ro52 in the context of epitopes, cell signalling and intracellular neutralisation of antibody bound pathogens, it remains unclear why it functions as an autoantigen in several autoimmune diseases (12, 28). Although no clear biochemical association of Ro52 and Ro60 has been demonstrated, the levels of their autoantibodies often follow each other (29). Whether temporal association or intermolecular epitope spreading as reported in mice explains this co-occurrence remains to be elucidated (30). A drawback of many clinical studies has been the use of the SSA/Ro complex containing both Ro52 and Ro60 in the analysis of autoantibodies rather than Ro52 *per se* (28). Since the BeSSTT cohort contains both mono-anti-Ro52 and mono-anti-Ro60 reactivities, we were able to demonstrate a greater contribution of anti-Ro52 and anti-SSB/La in glandular involvement and in peripheral signs of B-cell hyperactivity than of anti-Ro60 antibodies. This may be in line with Burbelo and colleagues who support a model whereby intracellular Ro52 bound antibody coated pathogen complexes, released or misprocessed from infected cells, drive autoantigenicity against Ro52 and the Fc region of IgG, known as RF (31). Besides evidence for a pathogenic role for anti-Ro52 antibodies in congenital heart block (32), passive transfer of anti-Ro52 immune sera into naïve mice induced salivary gland dysfunction implicating a direct role of the antibody in disease pathogenesis (33). However, it is not clear which mechanisms drive autoimmunity against exactly these three antigens, as epitope spreading over time in humans is difficult to demonstrate. Some data suggest that anti-SSB/La may come later (34).

Since autoantibodies can be present years before clinical disease (34) and antibodies to RNA binding proteins (Sm, RNP, SSA/Ro, SSB/La) tend to be stable over years (35, 36), we may speculate that subgroups with a potentially higher burden can be anticipated early or even before disease, where therapeutic intervention may have greatest chances of modifying disease progression and eventually outcome, as was already suggested (9). In this case, SGUS can be a tool to identify patients with glandular involvement at a preclinical stage (37).

This study benefitted from a well-defined cohort of patients who are included in the BeSSTT cohort because of suspicion for pSS based on the presence of at least one of the 2016 ACR/EULAR classification criteria. All serum samples were analysed in the same laboratory. In case of mono-reactivity, autoimmune serology was confirmed by another laboratory using a different ELISA or by repeatedly finding the same result over a longer period of time in our lab. An extensive and complete dataset was available for analysis. However, due to smaller numbers in the serology-based groups with mono-reactivities, analyses were underpowered to detect subtle differences between

mono-anti-Ro52 and mono-anti-Ro60 positive groups. Replication in a larger cohort is necessary to confirm these exploratory findings.

In conclusion, our results confirm that presence of anti-Ro60, anti-Ro52 and anti-SSB/LA autoantibodies in patients with a suspected or definite diagnosis of pSS influence the phenotypical presentation in an escalating manner with the least glandular disease expression in mono-anti-Ro60 positive patients, followed by the mono-anti-Ro52 and double anti-SSA/Ro positive, and most glandular involvement and signs of B-cell hyperactivity in triple positive patients. It can thus be concluded that individual detection of anti-Ro52, anti-Ro60 and anti-SSB/La reactivities is not only relevant in pSS diagnosis, but also provides insight in disease severity, potentially guiding specific personalised approach. We thus strongly recommend separate reporting of all three anti-Ro52, anti-Ro60 and anti-SSB/La antibodies in future research.

Take home messages
- Anti-Ro60, anti-Ro52 and anti-SSB/La reactivity profiles are associated with distinct phenotypes in pSS.
- Mono-anti-Ro60 positive patients displayed the least pSS features, those with triple reactivity against Ro60, Ro52 and SSB/La the most.
- Separate anti-Ro60, anti-Ro52 and anti-SSB/La detection is recommended in context of pSS diagnosis and phenotyping.

Acknowledgments

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