Clinical usefulness of anti-muscarinic type 3 receptor autoantibodies in patients with primary Sjögren's syndrome

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Abstract

Objective

To elucidate the clinical values of anti-M3R in Sjögren's syndrome (SS) in the largest cohort for an anti-M3R study.

Methods

The plasma of 361 subjects (156 primary SS [pSS], 62 non-SS-sicca [SICCA], 40 systemic lupus erythematosus [SLE], 50 rheumatoid arthritis [RA], and 53 healthy controls [HC]) was screened using our modified On-Cell-Western assay. Saliva from pSS (n=37) compared to SICCA (n=26) was also analysed. The sensitivity and specificity of anti-M3R and its association with comprehensive clinical and laboratory features were determined.

Results

Plasma-anti-M3R was higher in pSS compared to other groups, differentiating pSS with good-to-excellent diagnostic power with a specificity of 85% and a sensitivity between 75% and 98%. pSS plasma-anti-M3R was positively correlated with ocular staining scores, anti-Ro/SSA, IgG, β2-microglobulin, ESR, and ESSDAI. It was negatively correlated with WBC, C4, and salivary scintigraphic indices. Saliva-anti-M3R was 3.59 times higher in pSS than in SICCA. Interestingly, the agreement between the 2002 American European Consensus Group criteria and the criteria substituted with plasma-anti-M3R for the lip biopsy reached 92%, with a significant kappa of 0.824.

Conclusion

Anti-M3R enhances sensitivity and specificity for SS diagnosis, correlating with ocular dryness and glandular hypofunction, and the haematological/biological domains of the ESSDAI. Our findings also highlight the clinical significance of anti-M3R in SS diagnosis, especially where clinical assessments, such as lip biopsy, sialometry, or ocular evaluation, by multi-disciplinary specialists are limited.

Key words

Sjögren's syndrome, anti-muscarinic type 3 receptor autoantibodies, secretory dysfunction, anti-Ro/SSA, ESSDAI

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Received on April 1, 2020; accepted in revised form on June 22, 2020.

Funding: this study was supported by the National Institute of Dental and Craniofacial Research at the National Institute of Health [DE025726 and DE023838 to S. Cha, and T90DE021990 to M. Mona], and the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education [2015R1D1A1A01060468 to Y.J. Lee].

Competing interests: none declared.

Introduction

Sjögren's syndrome (SS) is an autoimmune disorder characterised by lymphocytic infiltration in the exocrine glands, leading to glandular dysfunction (1). Due to its heterogeneous clinical presentation, SS diagnosis remains a clinical challenge. Novel approaches to improve the specificity and sensitivity of current diagnostic tools are urgently needed (2). To date, autoantibodies against Ro/SSA have been the most used biological measures for SS diagnosis, as defined by the 2002 American-European Consensus Group (AECG) criteria and the 2016 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria (3, 4). Anti-Ro/SSA is known to be associated with systemic extraglandular manifestations, such as vasculitis, Raynaud's, arthritis, or renal tubular acidosis. However, its role in glandular dysfunction in SS has not been fully understood (5, 6).

Muscarinic-type-3-receptor (M3R), a G-protein-coupled acetylcholine receptor, is known to regulate secretion in salivary acinar cells (7). Out of the five subtypes of MR (M1R to M5R) (8), M3R is highly expressed in the exocrine glands and the M3R knockout mouse failed to induce saliva secretion (9). Previously, our group and others have reported that autoantibodies against M3R (anti-M3R) can suppress secretion from cells by functioning as an antagonist for the receptor (10-12). Jin *et al*. reported that incubation of cells with SS IgG significantly decreased M3R membrane localisation by inhibiting carbachol-induced intracellular calcium release.

The prevalence of anti-M3R is known to widely vary from 1.92% to 97% in SS, depending on the assay system (*i.e*. peptide-based ELISA versus cell-based assay) (13). Among studies with cell-based assays, anti-M3R was detected in 60% of SS patients by flow cytometry (14) and 75% of patients tested positive by our modified On-Cell Western (OCW) assay (15). Unlike conventional ELISA, these techniques allowed binding of autoantibodies to the conformational epitopes of M3R. Our previous study with the assay reported that anti-M3R IgG in plasma was highly prevalent in SS and that anti-M3R in combination with anti-Ro/SSA outperformed the single analyte in discriminating patients with SS from other groups (15). Moreover, the statistically significant correlation that existed between anti-M3R IgG and salivary flow rates/focus score in our previous analysis implied a potential role of anti-M3R in SS-disease parameters.

In this current study, we applied our in-house, modified OCW assay to screen plasma and saliva samples obtained from the Seoul National University Bundang Hospital (SNUBH) cohort, which is the largest cohort (n=361) for an anti-M3R study, to our knowledge. We aimed to determine the clinical/serological/laboratory characteristics of anti-M3R positive SS patients to determine clinical usefulness of anti-M3R. More importantly, we explored the potential clinical significance of anti-M3R in diagnosing SS by substituting minor salivary gland lip biopsy (MSGBx) with anti-M3R in the established SS classification criteria and evaluated its performance.

Patients and methods

Patient enrolment

Participants were recruited at SNUBH from August, 2005 to May, 2016. Primary SS patients (SS, n=156) were diagnosed according to the AECG criteria and patients with rheumatoid arthritis (RA, n=50) fulfilled the 2010 ACR/EULAR criteria (16). The 1997-updated criteria of the 1982-revised ACR criteria were used for systemic lupus erythematosus (SLE, n=40) (17). Non-SS-sicca group (Sicca, n=62) include subjects with dry mouth and/or dry eye but did not fulfill the AECG criteria. Gender- and age-matched healthy controls (HC, n=53) were enrolled from a routine medical check-up. This study was approved by the Institutional Review Board (B-0506/021-004) and the written informed consents were obtained.

Plasma and saliva collection

Collected blood tubes were centrifuged within 20 to 60 minutes of collection at 2,000 g for 10 min at 4°C. Plasma was separated, aliquoted into cryovials,

and stored at -70 °C until analysis. The whole saliva flow rate (WSFR) was determined, as previously described (18), after discontinuation of any xerostomic medication for at least four-fold its half-life. Briefly, unstimulated saliva was collected for 15 min, and stimulated whole saliva was collected for 5 min after the subjects chewed paraffin wax for 10s. The saliva volume was measured with a micropipette after centrifugation at 22,000 g for 10 min at 4°C and stored at -70°C until screening. Of 156 SS patients, 141 plasma samples (90.3%) and 36 unstimulated saliva samples (23%) without a protease inhibitor were screened after the samples were thawed on ice and briefly mixed on a vortex mixer.

Clinical parameters

Schirmer's test was performed in 93% (146/156) of SS patients and the average value from both eyes was used for analysis. Ocular staining score (OSS) was performed in 64% (101/156). The ocular surface was stained with a fluorescein strip wetted with buffered saline, and OSS was defined as the sum of staining scores of cornea, nasal conjunctiva, and temporal conjunctiva, which were evaluated by fluorescein sodium with a yellow filter (19). The staining scores of each area were graded as 0 (no staining), 1 (mild staining limited to <1/3 of the cornea), 2 (moderate staining of <1/2 of the cornea), or 3 (severe staining of >1/2 of the cornea). EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) was calculated as described (20) and moderate-to-high activity was defined as ESSDAI ≥5 (21).

In SS, the levels of WBC, Westergren erythrocyte sedimentation rate (ESR), C3 and C4, total IgG, and β2-microglobulin (B2M) were determined within 2 weeks of enrolment. MSGBx results were available in 66% (103/156) of SS patients and both the Chisholm-Mason score and focus score were calculated as previously reported (22, 23). Focus scores were categorised into 3 groups: low score group (focus score <1), intermediate score group (1≤focus score <2), and high score group (2≤ focus score). Salivary gland scintigraphy was performed on 69.8% (109/156) of SS patients. Scintigraphic parameters (parotid uptake ratio PU, submandibular uptake ratio SU, percentage parotid excretion % PE, and percentage submandibular excretion % SE) were calculated by using the region of interests around the frontal skull and salivary glands on anterior images (24). The uptake ratios and percentage excretions were calculated based on the means of right and left parametric values.

The modified OCW assay with the stable cell line expressing human M3R-GFP protein

As described in our previous study (15), stable HEK 293 cells expressing human M3R tagged with a green fluorescent protein (GFP) were seeded onto the 96-well plates (4 x 10^4 cells/well), followed by plasma (1:400 dilution) or saliva (1:1 dilution) incubation. Goat anti-human IgG (H+L) IRDye800CW secondary antibody (Rockland Immunochemicals, Inc.) at a dilution of 1:800 was used and the plate was screened at 800 nm wavelength on the Odyssey Reader (LI-COR Bioscience). The mean of at least three values was analysed. The signal intensities were analysed by the Odyssey software and normalised by GFP levels in each well, which was measured by a fluorescence microplate reader (BioTeck, 485/20 excitation and 528/20 emission), following our protocol (15).

Statistical analyses

Continuous variables are presented as mean (standard deviation [SD]) or median (interquartile range [IQR]), as appropriate. Group comparisons were performed with analysis of variance (ANOVA) followed by Bonferroni's post hoc tests or Kruskal-Wallis test followed by Dunn's post hoc tests. Receiver operating characteristic (ROC) curves were created to explore the ability of anti-M3R to distinguish SS from other groups, and area under the curve (AUC) with 95% confidence intervals (CI) were calculated. Comparison between AUCs was performed using the DeLong's test (package pROC). Optimum test cut-off values for anti-M3R intensity were based on maximum positive likelihood ratios (+LR) obtained from the ROC curve analysis (HC *vs*. SS). SS data were categorised into anti-M3R positive and negative, and compared to laboratory features using a Pearson χ^2 test or the Fisher's exact test, where appropriate. Spearman rank correlation coefficients were to assess associations between continuous variables. Cohen's kappa was to determine the level of agreement between the classification criteria. Prism v. 5.0 (GraphPad Software) and R (http://www.r-project.org, v. 3.5.1) in RStudio (http://www.rstudio.com, v. 1.1.456) were used. A *p*-value of less than 0.05 was considered significant.

Results

P-anti-M3R and S-anti-M3R are significantly upregulated in SS

The details of the SNUBH cohort are listed in Table I, including demographics and clinical features. The examples of wells indicate that the signal intensities of P-anti-M3R in SS plasma were markedly higher than in those detected in HC, Sicca, and RA ($p<0.001$) (Fig. 1A). It also reliably distinguished SS from HC (AUC 0.95, 95% CI 0.92 to 0.98), Sicca (AUC 0.95, 95% CI 0.91 to 0.98), or RA (AUC 0.89, 95% CI 0.84 to 0.94) ($p<0.0001$), while SS from SLE was less discriminatory (AUC 0.52, 95% CI 0.43 to 0.62) (Fig. 1B-C). The cut-off of 6.13% yielded a specificity of 85% for the discrimination of SS from HC, sicca, and RA, with a sensitivity of 98%, 95%, and 75%, respectively. Anti-M3R intensity in saliva (S-anti-M3R) was significantly different in SS compared to sicca ($p<0.0001$, Fig. 1D), with an AUC of 0.84 (AUC 0.95, 95% CI 0.92 to 0.98) (Fig. 1E). With a cut-off of 14.3% for S-anti-M3R, SS patients were identified with a specificity level of 98% and a sensitivity level of 50%.

P-anti-M3R positivity is associated with anti-Ro/SSA and glandular infiltration

SS patients were categorised into anti-M3R positive and negative groups (Table II) based on the cut-off value of HC mean ± 2SD. SS patients with positive P-anti-M3R were significantly associated with anti-Ro/SSA and anti-La/

Table I. Demographic and clinical characteristics of the SNUBH study cohort.

	HC (n=53)	Sicca (n=62)	SS (n=156)	SLE (n=40)	RA (n=50)
Age, years	50.26 ± 12.81	52.42 ± 11.72	50.13 ± 12.75	32.98 ± 12.26	50.38 ± 12.67
Female Sex-	51/53 (96.2)	58/62 (93.5)	152/156 (97.4)	30/40 (75)	47/50 (94)
WBC (/mm^3)	5654 ± 1356	5791 ± 1492	4974 ± 1607	4130 ± 2651	6899 ± 2244
Hb (g/dL)	13.6 ± 1.0	13.3 ± 1.1	12.6 ± 1.3	11.2 ± 2.3	12.5 ± 1.3
Platelet (/mm^3)	260.3 ± 4.7	242.5 ± 57.0	229.1 ± 65.2	182.9 ± 111.4	273.8 ± 65.7
ESR (mm/h)	9.0 ± 7.6	11.2 ± 11.2	24.5 ± 20.5	33.6 ± 24.8	19.1 ± 22.5
C3 (mg/dL)	ND	94.2 ± 19.0	105.7 ± 24.0	56.2 ± 30.1	ND
C4 (mg/dL)	ND	21.6 ± 8.0	22.1 ± 10.6	11.5 ± 6.8	ND
B2M (mg/L)	ND	1.9 ± 0.6	2.4 ± 1.3	ND	ND
IgG (mg/dL)	ND	1326.4 ± 278.7	1998.0 ± 673.7	ND	ND
Anti-SSA+	ND	3/58 (5.2)	141/156 (90.4)	25/34 (73.5)	ND
Anti-SSB+	ND	0/58 (0.0)	79/156 (50.6)	10/34 (29.4)	ND
Lip biopsy	ND	46/62 (74.2)	103/156 (66.0)	ND	ND
Focus score+	ND	1/46 (1.2)	83/103 (80.6)	ND	ND
Chisholm-Mason Scale >1	ND	2/46 (4.3)	87/103 (84.5)	ND	ND
Avg Schirmer (mm)	ND	10.1 ± 7.1	7.3 ± 6.9	ND	ND
Unstimulated salivary flow rate (mL/min.)	ND	0.266 ± 0.0.562	0.081 ± 0.102	ND	ND
PU ratio	ND	5.73 ± 2.69	4.10 ± 1.66	ND	ND
SU ratio	ND	6.44 ± 1.48	4.57 ± 1.41	ND	ND
%PE	ND	38.6 ± 17.3	27.5 ± 20.6	ND	ND
%SE	ND	30.1 ± 14.0	16.4 ± 14.4	ND	ND
Lacrimal dysfunction	ND	26/55 (47.3)	125/155 (80.6)	ND	ND
Salivary dysfunction	ND	38/60 (63.3)	148/155 (95.5)	ND	ND

Data are presented as mean ± SD or positive individual/total number (percentage).
ND: not determined.

SSB (p<0.001). Additionally, P-anti-M3R positive patients were more likely to have a high grade of mononuclear cell infiltration in the minor salivary glands (p<0.05) and a high level of total IgG (p<0.05). Interestingly, a higher percentage of SS patients with positive P-anti-M3R shows a tendency toward Raynaud's phenomenon (26.5% *vs*. 8%, p=0.065) or leukopenia (32.5% *vs*. 12.5%, p=0.052) compared to those with a negative result. As shown in Supplementary Table S1, S-anti-M3R positive SS patients also had a significantly higher prevalence of anti-Ro/SSA (100% *vs*. 76.9%, p=0.040). It is of note that S-anti-M3R was prevalent in those with unstimulated WSFR ≤0.1 mL/min (87.0% *vs*. 53.8%, p=0.046).

Anti-M3R is correlated with SS autoantibodies, scintigraphy parameters, and WSFR, analysed by the bivariate correlation analysis

Anti-M3R was analysed for its correlation with haematoimmunological parameters by the bivariate correlation test (Table III). P-anti-M3R and S-anti-M3R were correlated with each other, and both were also correlated with anti-Ro/SSA or anti-La/SSB. Although anti-Ro/SSA and anti-La/SSB correlated negatively with age, anti-M3R was not affected by age. P-anti-M3R positively correlated with B2M (R=0.38, p<0.0001) or total IgG (R=0.42, p<0.0001), ESR (R=0.39, p<0.0001), ESSDAI (R=0.24, p=0.004), focus score (R=0.56, p<0.0001), and average OSS (R=0.28, p<0.05). P-anti-M3R correlated negatively with C4 levels (R=-0.20, p<0.05), unstimulated WSFR (R=-0.260, p<0.01), stimulated WSFR (R=-0.286, p<0.01), and WBC (R=-0.34, p<0.0001). This analysis was also strongly supported by our regression analysis, as shown in Supplementary Figure S1.

The relationship between anti-M3R with salivary glandular excretion was determined by analysing scintigraphic parameters and WSFR (Table III). P-anti-M3R was found to be inversely proportional to ExSM (the percentage of submandibular excretory function, %SE) and ExP (parotid gland excretory function, %PE) measured by ^{99m}Tc-pertechnetate salivary gland scintigraphy (R=-0.386 and R=-0.378, respectively, p<0.0001). It was also negatively correlated with unstimulated and stimulated WSFR (R=-0.260 and R=-0.286, respectively, p<0.0001). We also found that P-anti-M3R was correlated with parotid gland uptake ratio of the radioactive tracer, PU (R=0.346, p<0.0001) and submandibular gland uptake of the tracer, SMU (R=-0.346 and R=-0.431, respectively, p<0.0001). In addition, S-anti M3R was also found to be associated with these scintigraphic parameters (except for %PE) and WSFR.

P-anti-M3R is associated with scintigraphy parameters and OSS while S-anti-M3R is associated with WSFR, analysed by the regression analysis

Supplementary Figure S2 presents linear relations analysed by the regression analysis. P-anti-M3R demonstrated a linear relation with ExSM and ExP (Fig. S2A-B). S-anti-M3R showed phase-one decay association with ExSM and ExP (Fig. S2E-F). Stimulated and unstimulated WSFR showed a linear relationship with S-anti-M3R (p<0.05) (Fig. S2G-H).

We also included ocular test results to analyse their association with anti-M3R. OSS, a diagnostic measure for dry eyes, was directly correlated with P-anti-M3R by the linear regression analysis (p<0.05, Fig. S2I) while

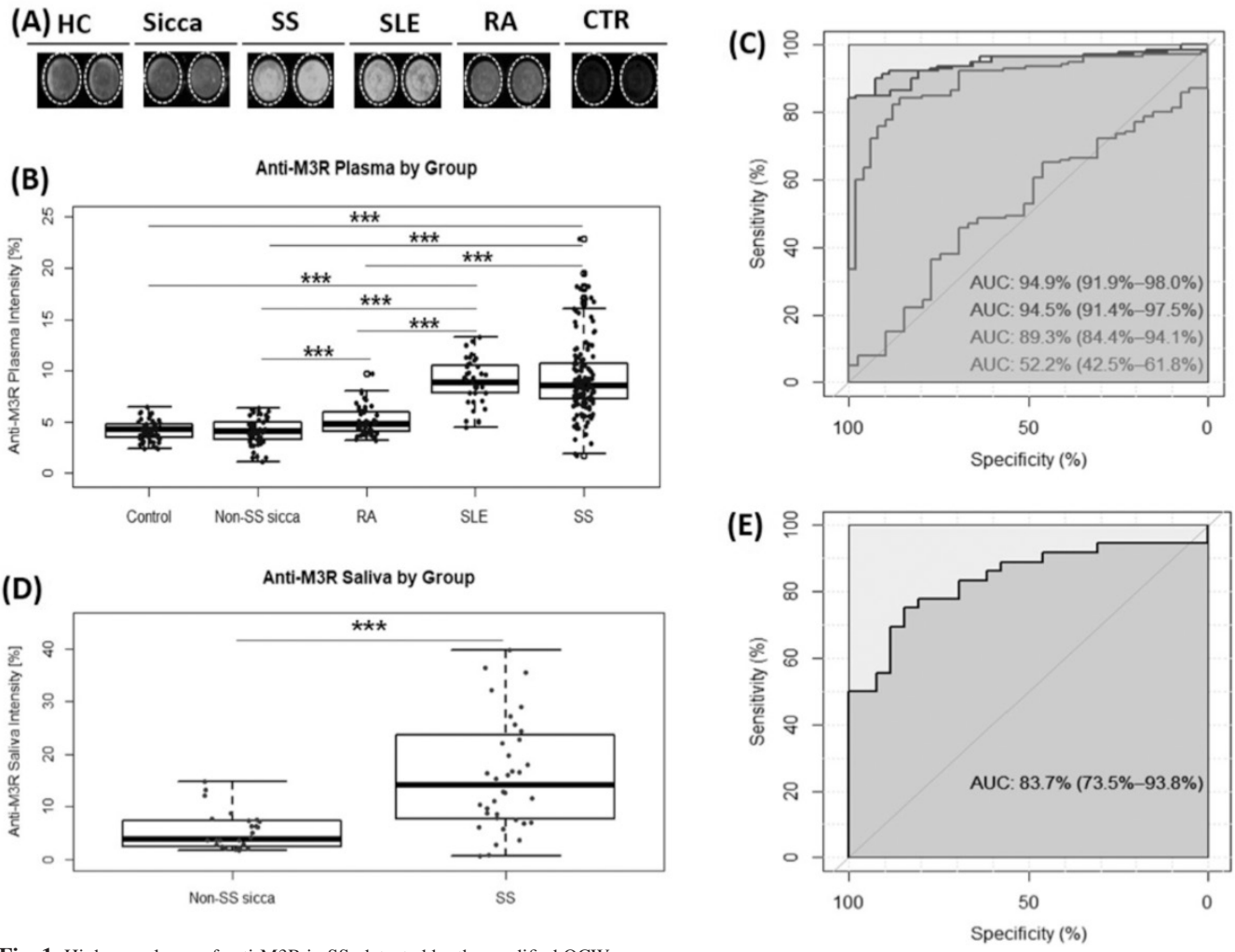

Fig. 1. High prevalence of anti-M3R in SS, detected by the modified OCW assay.
A: Representative OCW images of P-anti-M3R in HC (n=53), Sicca (n=62), SS (n=156), SLE (n=40) and RA (n=50). Control (CTR) is a negative control (2°Ab only). SS and SLE visibly showed higher levels of P-anti-M3R intensity (white shade) as compared to other groups.
B, **D**: Box-and-whisker plots of anti-M3R intensity in plasma (A) and in saliva (D). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate 1.5 times the interquartile range from either end of each box and circles represent outliers. Each individual value is plotted as a dot superimposed on the graph. Kruskal-Wallis test followed by Dunn's posttest (correction for multiple testing) was applied. Significant differences are indicated as *** (p<0.001).
C: Receiver operating characteristic curves (ROC) of P-anti-M3R for distinguishing patients with SS from HC (the first black line), Sicca (the second line), RA (the third line) and SLE (the fourth line). (E) ROC curve of anti-M3R in saliva for discrimination between SS and Sicca patients.

S-anti-M3R shows one-phase decay association (Fig. S2J). The average Schirmer's test showed a tendency of linear relationship with P-anti-M3R (Fig. S3K) or one-phase decay association with S-anti-M3R (Fig. S2L). Since anti-M3R may affect both eyes, we applied the average of the two values to these analyses in addition to the worst value as suggested by the SS diagnostic criteria (Table III).

The diagnostic performance of the 2002 AECG criteria for SS improved when substituted with P-anti-M3R for histopathology

To determine the value of anti-M3R in SS diagnosis, the item of focus score ≥1 in the minor salivary gland in the 2002 AECG criteria system was replaced with P-anti-M3R (Table IV). The data analysis indicates that P-anti-M3R was significantly correlated with the number of the 2002 AECG criteria satisfied (n=125, R=0.540, p=7.847×10^{-11}) and the score of 2016 ACR/EULAR criteria (n=193, R=0.579, p=1.000×10^{-13}). When compared to the original AECG criteria or the 2016 ACR/EULAR criteria, our substituted classification criteria showed a substantial agreement of 92.8% (Cohen's κ=0.824) and 90.7% (κ=0.779), respectively. When using the 2002 AECG criteria as the reference, the estimated sensitivity and specificity of the substituted criteria were 92.9% and 92.5%, respectively, with a positive likelihood ratio of 12.31. When the performance of the substituted criteria was analysed based on the 2016 ACR/EULAR criteria, the sensitivity and specificity were 92.7% and 86%, respectively, with a positive likelihood ratio of 6.60.

Discussion

As SS-specific biomarkers are unavailable to date, SS diagnosis requires the measurement of multiple clinical parameters, including less invasive blood tests to invasive MSGBx (25).

Table II. Comparison between P-anti-M3R positive and negative SS patients.

	Plasma anti-M3R (-), n=24	Plasma anti-M3R (+), n=117	*p*-value
Ocular symptoms*	20 (83.3%)	93 (79.5%)	0.785
Ocular signs*	17 (70.8%)	95 (81.9%)	0.217
Schirmer test ≤5 mm	13/23 (56.5%)	68/103 (66.0%)	0.390
OSS ≥5	5/18 (27.8%)	24/71 (33.8%)	0.626
Oral symptoms*	21 (87.5%)	101 (86.3%)	1.000
Salivary gland involvement*	23 (95.8%)	111/116 (95.7%)	1.000
Unstimulated WSFR ≤0.1 mL/min	17/23 (73.9%)	89/110 (80.9%)	0.448
Abnormal salivary scintigraphy	16/18 (88.9%)	95/107 (88.8%)	1.000
Anti-Ro/SSA (+)	14 (58.3%)	114 (97.4%)	**6.672×10^{-7}**
Anti-La/SSB (+)	4 (16.7%)	65 (55.6%)	**0.001**
Focus score ≥1	13/19 (68.4%)	59/69 (85.5%)	0.087
Focus score ≥2	4/19 (21.1%)	33/69 (47.8%)	**0.042**
Chisholm–Mason grade = 4	5/19 (26.3%)	41/69 (59.4%)	**0.011**
ESSDAI ≥5	2 (8.3%)	20 (17.1%)	0.368
Extraglandular manifestations	12 (50.0%)	65 (55.6%)	0.619
Raynaud's phenomenon	2 (8.3%)	31 (26.5%)	0.065
Leukopenia	3 (12.5%)	38 (32.5%)	0.052
Hypocomplementaemia C3 or C4	1/23 (4.3%)	12/115 (10.4%)	0.695
Total IgG ≥1700 mg/dL	10/23 (43.5%)	79 (67.5%)	**0.028**

p-values were calculated by chi-square or Fisher's exact test as applicable.
*defined according to the 2002 AECG classification criteria.

The pathological role of anti-Ro/SSA in hyposalivation is unclear, although its association with extraglandular manifestations is relatively well accepted (6, 26). Anti-M3R prevalence in SS varies from 1.9% to 97.0%. Most studies on anti-M3R evaluated a small sample size (n<60 in 14 out of 22 studies), mainly utilising a linear peptide-based ELISA (13). When compared with controls, the prevalence of anti-M3R in SS was significantly higher in 11 studies whereas the other 11 studies showed no significant difference (13). Interistingly, by maintaining M3R tertiary structure, our laboratory and others have consistently reported upregulation of anti-M3R expression in SS patients (14, 27). These studies clearly indicate that anti-M3R requires a detection method designed for conformation-dependent epitopes, which has challenged the establishment and calibration of cell-based assays for anti-M3R screening.

The modified OCW assay has allowed our group to perform a reliable anti-M3R screening method with plasma samples from the UF cohort (15). In the UF study, anti-M3R levels were significantly elevated in SS plasma in comparison with HC, SLE, or RA ($p<0.01$). Furthermore, anti-M3R was associated with anti-Ro/SSA positivity ($p=0.035$), and indicated positive linear associations with the focus score ($p<0.01$) and negative associations with its unstimulated WSFR ($p<0.05$) (15). In our current study with the largest SNUBH cohort for an anti-M3R study, we found that P-anti-M3R was significantly elevated in SS, reliably distinguishing SS from other conditions. AUC has shown reliability of P-anti-M3R of 96.9%, 94.5%, and 89.3% in distinguishing SS from HC, Sicca, and RA, respectively. In addition, S-anti-M3R showed reliability of 83% in distinguishing SS from sicca, based on the presence of anti-M3R IgG, and the detection of anti-M3R IgA might improve the reliability. The cut-off of 6.13 for positive anti-M3R in the unadsorbed, deidentified SNUBH cohort samples was almost identical to the cut-off of 6.24 in our previous study with UF samples (15), indicating the reliability of our cell-based assay.

Interestingly, P-anti-M3R does not substantially distinguish SS from SLE (AUC of 0.56), unlike in the UF study (AUC of 0.72) (15). Potential reasons include: 1) SLE and SS patients demonstrate some common and/or overlapping clinical/serological features, which makes SS diagnosis challenging (28, 29). 2) Ethnically heterogeneous (UF) and homogeneous (SNUBH) groups were enrolled for the previous and the current study, respectively. A different prevalence of anti-M3R in different ethnic groups with SS or SLE can be presumed. 3) Notably, the number of anti-Ro/SSA-positive SLE patients at SNUBH is significantly higher than SLE patients at UF (74.3% *vs*. 38.9%). In addition, almost all SNUBH patients tested positive for anti-nuclear antibody in SS and SLE patients whereas the SS and SLE patients at UF were 80% and 72% positive, respectively (data not shown). It has been reported that anti-Ro/SSA is more commonly detected in Asian SLE patients, including Koreans, than in Caucasians (30, 31). A stringent inclusion and exclusion criteria may minimise differences in subject eligibility among various facilities when multicenter studies are designed.

We also found that S-anti-M3R is significantly higher in SS than in sicca. Screening of S-anti-M3R required a 1:1 dilution and a cut-off of 14.3 for positivity while P-anti-M3R required a 1:400 dilution with a cut-off of 6.13. Major antibody classes in saliva are secretory or polymeric IgA (sIgA) and IgG. Unlike sIgA, which is mainly synthesised by plasma cells in the salivary gland and secreted by receptor-mediated transcytosis, most of salivary IgG are derived from serum by passive diffusion through gingival crevices (32). Although salivary glandular/gingival plasma cells may produce salivary IgG, its concentration is much lower than that in serum (33). Therefore, this low sensitivity of detecting S-anti-M3R IgG is unsurprising. Despite its low concentration, salivary IgG levels are known to be correlated with serum IgG levels, reflecting systemic immunity (32). We were unable to determine whether anti-M3R sIgA is elevated in SS due to unavailability of the OCW compatible-IRDye®800CW-anti-IgA secondary antibody on the market. Future analyses of saliva, depending upon the availability of the antibody, may enhance detection sensitivity, and will provide a clear insight into the prevalence and usefulness of S-anti-M3R as a non-invasive diagnostic tool for SS.

Our analysis found that P-anti-M3R

Table III. Bivariate correlation analysis of SS autoantibodies with clinical parameters in subjects with pSS or Sicca.

	Plasma anti-M3R	Salivary anti-M3R	Anti-Ro/SSA	Anti-La/SSB
Average Schirmer's	-0.145 (0.055)/175	-0.237 (0.163)/52	**-0.143 (3.52×10^{-4})/190**	**-0.169 (0.026)/190**
Worst Schirmer's	-0.118 (0.120)/175	-0.245 (0.080)/52	**-0.225 (0.002)/190**	-0.115 (0.190)/190
Average OSSs	**0.283 (0.020)/121**	**0.519 (0.047)/27**	**0.348 (9.18×10^{-5})/133**	**0.315 (4.25×10^{-4})/133**
Worst OSSs	**0.292 (0.001)/121**	**0.540 (0.004)/27**	**0.333 (8.83×10^{-5})/133**	**0.338 (7.06×10^{-5})/133**
USWSFR	**-0.260 (3.89×10^{-4})/183**	**-0.512 (0.001)/62**	**-0.364 (4.80×10^{-7})/202**	**-0.256 (4.79×10^{-4})/202**
SWSFR	**-0.286 (8.75×10^{-5})/183**	**-0.512 (4.07×10^{-4})/62**	**-4.09 (1.11×10^{-8})/202**	**-0.347 (1.55×10^{-6})/202**
Focus score*	**0.562 (4.02×10^{-12})/129**	**0.569 (0.001)/48**	**0.549 (1.91×10^{-11})/148**	**0.444 (1.34×10^{-7})/148**
PU	**-0.346 (1.23×10^{-4})/118**	**-0.586 (0.007)/41**	**-0.431 (1.22×10^{-6})/134**	**-0.431 (1.23×10^{-6})/134**
SMU	**-0.431 (1.08×10^{-6})/118**	**-0.598 (0.005)/41**	**-0.517 (2.92×10^{-9})/134**	**-0.429 (1.37×10^{-6})/134**
%PE	**-0.386 (1.11×10^{-5})/122**	-0.400 (0.081)/41	**-0.398 (6.86×10^{-6})/138**	**-0.435 (6.12×10^{-7})/138**
%SE	**-0.378 (1.94×10^{-5})/121**	**-0.489 (0.029)/41**	**-0.442 (4.71×10^{-7})/138**	**-0.339 (1.52×10^{-4})/138**
Age	-0.136 (0.060)/193	-0.136 (0.416)/62	**-0.143 (0.049)/211**	**-0.254 (3.81×10^{-4})/211**
WBC	**-0.341 (1.24×10^{-6})/193**	**-0.330 (0.043)/62**	**-0.275 (1.26×10^{-4})/211**	**-0.298 (2.83×10^{-5})/211**
Hb	**-0.263 (2.25×10^{-4})/193**	**-0.330 (0.047)/62**	**-0.184 (1.26×10^{-4})/211**	**-0.182 (0.012)/211**
Platelet	**-0.201 (0.005)/193**	0.125 (0.455)/62	-0.127 (0.080)/211	0.018 (0.805)/211
ESR	**0.389 (2.19×10^{-8})/193**	**0.545 (4.01×10^{-4})/62**	**0.361 (2.99×10^{-7})/211**	**0.459 (2.52×10^{-11})/211**
C4	**-0.204 (0.011)/155**	-0.144 (0.503)/37	-0.021 (0.799)/166	-0.052 (0.524)/166
Total IgG	**0.416 (6.70×10^{-8})/156**	**0.439 (0.036)/37**	**0.407 (1.81×10^{-7})/168**	**0.488 (1.34×10^{-10})/168**
B2M	**0.379 (1.63×10^{-6})/151**	**0.542 (0.009)/32**	**0.343 (2.00×10^{-5})/159**	**0.332 (3.55×10^{-5})/159**
ESSDAI	**0.242 (0.004)/141**	0.229 (0.319)/36	**0.320 (1.30×10^{-4})/154**	**0.308 (2.26×10^{-4})/154**
Plasma anti-M3R		**0.620 (3.27×10^{-5})/38**	**0.737 (1.00×10^{-13})/190**	**0.536 (1.02×10^{-13})/190**
Salivary anti-M3R	**0.620 (3.27×10^{-5})/38**		**0.781 (1.22×10^{-8})/58**	**0.608 (5.09×10^{-5})/58**

OSS: ocular staining score; USWSFR: unstimulated whole salivary flow rate; SWSFR: stimulated whole salivary flow rate; PU: parotid gland uptake in the salivary scintigraphy; SMU: submandibular gland uptake in the salivary scintigraphy; %PE: percentage parotid excretion in the salivary scintigraphy; %SE: percentage submandibular excretion in the salivary scintigraphy; WBC: white blood cells; Hb: haemoglobin; ESR: erythrocyte sedimentation rate; B2M: β2 microglobulin; ESSDAI: EULAR Sjögren's syndrome disease activity index.
*stratified into 3 groups (focus score <1, 1≤focus score <2, and 2≤ focus score). Numbers indicate "correlation coefficient (*p*-value)/sample size".

Table IV. The diagnostic performance of anti-M3R in place of histopathology in the SS criteria.

Analysed criteria Reference criteria	P-anti-M3R substituted criteria 2002 AECG criteria	P-anti-M3R substituted criteria 2016 ACR/EULAR criteria	2016 ACR/EULAR criteria 2002 AECG criteria
No. of observed agreement	180 (92.78%)	179 (90.72%)	188 (96.91%)
Kappa	0.824 (0.736-0.913)*	0.779 (0.682-0.876)	0.924 (0.864-0.984)
Sensitivity	92.91% (87.34-96.55)	92.70% (86.99-96.44%)	96.45% (91.92-98.84)
Specificity	92.45% (81.79-97.91%)	85.96% (74.21-93.74%)	98.11 (89.93-99.95)
Positive likelihood ratio	12.31 (4.79-31.62)	6.60 (3.47-12.58)	51.12 (7.33-356.33)
Negative likelihood ratio	0.08 (0.04-0.14)	0.08 (0.05-0.16)	0.04 (0.02-0.09)

In the substituted classification criteria, the item of histopathology (lip biopsy) in the 2002 AECG criteria was replaced with plasma anti-M3R; n=194; *95% confidential interval.

was significantly associated with important SS-disease parameters, such as anti-Ro/SSA, focus score ≥2, grade 4 on the Chisholm-Mason scale, and hypergammaglobulinaemia. S-anti-M3R positivity was associated with anti-Ro/SSA and unstimulated WSFR. In addition, our bivariate correlation analysis suggested the potential involvement of P-anti-M3R in extraglandular manifestations, shown as a negative correlation with WBC and platelet. Autoimmune cytopenia is a well-known extraglandular manifestation of SS. A study has shown that anti-M3R enhanced Jurkat T cell death through MHC class I down-regulation when co-incubated with NK cells (34). Notably, P-anti-M3R levels were negatively correlated with serum C4 levels in our study, whereas anti-Ro/SSA showed no correlation. In SS, low C4 or C3 is observed in about 15% of SS patients (35) and is considered to be a marker for systemic manifestations and mortality in SS (36). Importantly, low C4 is known to be a risk factor for non-Hodgkin lymphoma in SS (37). Hypergammaglobulinaemia was also positively associated with P-anti-M3R. Taken together, P-anti-M3R was significantly correlated with ESSDAI in our SS cohort, especially for the haematological and biological domains. Interestingly, anti-M3R showed a tendency toward a positive association with Raynaud's phenomenon in our study. No direct evidence is available in the literature for the roles of anti-M3R in Raynaud's phenomenon. However, a recent study with the M3R knock-out mouse model demonstrating impaired vasodilation (38), suggests a potential role of anti-M3R in impaired vasodilation, which warrants further investigation.

The histopathology is a key measure in the 2002 and 2016 criteria, as focus scores are generally considered to be a robust tool for SS diagnosis. However, focal lymphocytic infiltration was observed in 15% of healthy volunteers

without a history of salivary gland dysfunction (39). Furthermore, inter-observer and intraobserver agreements in MSGBx were 0.71 and 0.76, respectively, and many pathologists failed to delineate focal lymphocytic sialadenitis from non-specific lymphocytic sialadenitis in a multicenter study (40). In a longitudinal study where MSGBx was repeated after 2 to 3 years, the results changed from focus-negative to focus-positive in 7% of participants and from focus-positive to focus-negative in 11% of subjects (41). Furthermore, this invasive procedure led to lower lip paresthesia in 6% of subjects (42). Due to such limitations, salivary gland ultrasonography has recently been proposed as a substitution for MSGBx despite the key roles of the biopsy in diagnosis, research, stratification of patients, prediction of lymphoma, and therapeutic development. A recent systematic review has shown the sensitivity of ultrasonography ranged from 45.8 to 91.6% and specificity from 73 to 98.1% (43). When compared to the 2002 AECG classification criteria, Mossel *et al*. reported an agreement of 82%, sensitivity of 71%, and specificity of 92% (44), and Takagi *et al*. reported a sensitivity of 81% and specificity of 86% (45). Therefore, higher sensitivity (92.91%) and specificity (92.45%) of P-anti-M3R in the substituted 2002 AECG criteria than those of ultrasonography highlight anti-M3R as a specific and sensitive biomarker in diagnosing this complex disease.

In conclusion, the dissemination of P-anti-M3R screening assay and the development of a non-invasive liquid biopsy involving S-anti-M3R could lead to an amelioration of our current way of approaching the SS diagnosis. Anti-M3R, in fact, was highly prevalent in SS patients compared to controls and significantly related to glandular infiltration, impaired exocrine function, and disease activity, showing interesting relationships mainly with haematological and biological parameters of disease. Overall, anti-M3R potentially represents a novel pathogenetic biomarker of SS, linking inflammation, autoimmunity, glandular dysfunction and, potentially, lymphoproliferation.

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