ImmunoHorizons

RESEARCH ARTICLE

Clinical and Translational Immunology

Immunoassay Detects Salivary Anti-SSA/Ro-52 Autoantibodies in Seronegative Patients with Primary Sjögren's Syndrome

Sarah Kamounah,[*,†,1] Nabihah Tayob,[‡,§,1] Samantha Chiang,[*,1] Fang Wei,[*,1] Jin Kyun Park,[¶] Hyun Mi Kwon,[¶] Ziding Feng,[‖] David Chia,[#] Anne Marie Lynge Pedersen,[†] Yeong Wook Song,[¶] and David T.W. Wong*

*Center of Oral/Head & Neck Oncology Research, School of Dentistry, University of California, Los Angeles, Los Angeles, CA; [†]Department of Odontology, Section of Oral Biology and Immunopathology/Oral Medicine & Pathology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen N, Denmark; [‡]Department of Data Science, Dana-Farber Cancer Institute, Boston, MA; [§]Department of Medicine, Harvard Medical School, Boston, MA; [¶]Division of Rheumatology, Department of Internal Medicine, Seoul National University College of Medicine, Seoul, South Korea; [‖]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA; and [#]Department of Pathology & Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA

ABSTRACT

The diagnostic work-up for Sjögren's syndrome is challenging and complex, including testing for serum autoantibodies to SSA/Ro and a labial salivary gland biopsy. Furthermore, the diagnosis is often delayed. In this study, we tested the hypothesis that anti-SSA/Ro autoantibodies are detectable in the saliva of patients with primary Sjögren's syndrome (pSS) because the disease affects the salivary glands, and these autoantibodies display greater discriminatory performance in saliva than in serum. SSA/Ro-52 Ags were used to develop what is, to our knowledge, a novel quantitative electrochemical-based immunoassay: the electric field–induced release and measurement (EFIRM) platform. The clinical utility was determined by measuring salivary anti-SSA/Ro-52 autoantibodies in patients with pSS and sicca ($n = 34$), patients without pSS with sicca ($n = 35$), and healthy subjects ($n = 41$). The statistical analysis of discrimination included the area under the receiver operating characteristic curve. Salivary anti-SSA/Ro-52 autoantibodies were measured in 94% (32 of 34) of patients with pSS with 85% (29 of 34) seropositivity. Four of the five seronegative patients with pSS had EFIRM-measurable anti-SSA/Ro-52 autoantibodies in saliva. Additionally, 60% (21 of 35) of the seronegative patients without pSS who had sicca had EFIRM-detectable SSA/Ro-52 autoantibodies in saliva, indicating the onset of autoimmune disease. Two of the 41 healthy control subjects had EFIRM-detectable SSA/Ro-52 autoantibodies in their saliva. Salivary SSA/Ro-52 autoantibodies significantly discriminated patients with pSS or patients with the initial stage of autoimmune disease from healthy subjects with an area under the receiver operating characteristic curve of 0.91. Our findings suggest that the proposed saliva SSA/Ro-52 immunoassay improves early and accurate diagnosis of seronegative patients with pSS and patients with early-onset autoimmune disease. *ImmunoHorizons*, 2023, 7: 554–561.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic chronic autoimmune disease characterized by focal lymphocytic infiltration of the exocrine glands, including salivary and lacrimal glands, leading to oral and ocular dryness (1–3). Extraglandular manifestations include constitutional symptoms (e.g., fatigue, myalgia, and arthralgia), glomerulonephritis, and vasculitis-induced

Received for publication June 29, 2023. Accepted for publication June 29, 2023.

Address correspondence and reprint requests to: David T.W. Wong, Center for Oral/Head and Neck Oncology Research, School of Dentistry, University of California, Los Angeles, 10833 Le Conte Avenue, CHS 73-041, Los Angeles, CA 90095. E-mail address: dtww@g.ucla.edu

[1]S.K., N.T., S.C., and F.W. contributed equally to this work.

ORCIDs: 0000-0002-9019-3322 (F.W.); 0000-0002-0290-2206 (D.T.W.W.).

This work was supported by the National Institutes of Health (DE017593) by a Sjögren's Syndrome Foundation High Impact Research Award; and by grants from the Independent Research Fund of Denmark and the Danish Dental Association Foundation; from the Ministry of Science, ICT, and Future Planning (NRF-2015M3A9B6052011, 2019M3A9A8065574); and from the National Institute of Dental and Craniofacial Research at the National Institutes of Health (DE030990).

Abbreviations used in this article: ACR, American College of Rheumatology; ANA, antinuclear Ab; AUC, area under the receiver operating characteristic curve; CI, confidence interval; EFIRM, electric field–induced release and measurement; IRB, institutional review board; pSS, primary Sjögren's syndrome; ROC, receiver operating characteristic.

The online version of this article contains supplemental material.

https://doi.org/10.4049/immunohorizons.2300043

ImmunoHorizons is published by The American Association of Immunologists, Inc.

rash or peripheral neuropathy, as well as B-cell lymphoma as a rare but serious complication (3, 4). Diagnosis of pSS can be challenging. About 30% of the cases of mucosal dryness are believed to occur because of age-related atrophy of the exocrine gland tissue leading to glandular hypofunction or because of intake of medication (5). The diagnosis of pSS is complex and requires the detection of anti-SSA/Ro autoantibodies in serum and/or focal lymphocytic infiltration in minor labial salivary gland biopsies as the main criteria to support the diagnosis (3).

In pSS, chronic inflammation of the glandular tissue is mediated by abnormal T and B cell responses to autoantigens such as SSA/Ro and SSB/La (4). Previous findings have indicated that the epithelial cells of the exocrine glands are not merely targets of infiltrating immune cells but are actively involved in the autoimmune response (6, 7). Because the salivary gland epithelium possibly provides SSA/Ro autoantigen to the infiltrating T and B cells, the locally produced SSA/Ro autoantibodies will be secreted into the saliva before they appear in the blood circulation. Previous studies have detected salivary anti-SSA in seropositive patients with pSS with sicca but not in patients without pSS with sicca (8–10). To our knowledge, there is currently no reliable assay that has quantitatively detected anti-SSA/Ro autoantibodies in saliva from seronegative pSS patients and non-pSS, sicca patients (11, 12).

It has previously been reported that autoantibodies are present in serum as early as 20 y before the definitive diagnosis of pSS is made, and anti-SSA/Ro-52 has the highest positive predictive value in the early onset of pSS and the severity of the disease course (13). It would be invaluable to improve earlier clinical diagnosis of pSS to possibly initiate therapeutic intervention at an earlier stage, including measures to prevent damaging local effects of exocrine dysfunction, although this is not standard practice yet. We aim to improve early pSS diagnosis by using what is, to our knowledge, a novel electrochemical immunoassay, electric field–induced release and measurement (EFIRM), for salivary anti-SSA/Ro-52 autoantibody detection. EFIRM is based on an electrochemical sensing technology that uses changes in an electric field to detect Abs, circulating DNAs, and RNAs in saliva and plasma. EFIRM has sensitivity exceeding that of ELISA, allowing detection of saliva and plasma Abs to SARS-CoV-2 spike protein in infectious and recovered patients that have a 100-fold difference in concentration (14). The aims of this study were to develop (to our knowledge) a novel EFIRM-based analytic platform that can quantitatively measure anti-SSA/Ro-52 autoantibodies in saliva and to determine whether the saliva-based immunoassay can improve the detection of anti-SSA/Ro-52 autoantibodies in seronegative patients with pSS and patients with early onset of the disease for early screening.

MATERIALS AND METHODS

Patients

This prospective cohort included 34 patients fulfilling the American College of Rheumatology (ACR) classification criteria for Sjögren's syndrome (15) and 35 patients who had Sjögren-like symptoms but did not fulfill the classification criteria for pSS (designated "non-pSS sicca"). All patients were evaluated at the rheumatology clinic at Seoul National University Hospital for diagnostic work-up. Forty-one age- and sex-matched healthy subjects with no history of autoimmune disease were included. The study was approved by the institutional review boards (IRBs) at University of California, Los Angeles (IRB no. 13-001075), and Seoul National University Hospital (IRB no. 1302-068-464). Written informed consent was obtained from all participants.

Saliva collection

Unstimulated whole saliva samples were collected for 15 min as previously described (16, 17). The samples were kept on ice and centrifuged immediately after collection at 2600 × *g* for 15 min at 4°C. The supernatant was supplemented with a protease inhibitor mixture consisting of 1 μl aprotinin (stock 10 mg/ml; Sigma-Aldrich Corp., St. Louis, MO), 3 μl Na_3VO_4 (stock 400 mM; Fivephoton Biochemicals, San Diego, CA), and 10 μl PMSF (stock 10 mg/ml; Sigma-Aldrich Corp.) and stored at −80°C until analysis. For analysis, the saliva samples were thawed and vortexed for 10 s.

Detection of serum anti-SSA/Ro

IgG-class Abs to SSA/Ro in serum were measured using ELISAs (Zeus Scientific, Branchburg, NJ) at the time of routine diagnostic work-up for pSS.

EFIRM immunoassay

All experimental work for electropolymerization and electrochemical readout was performed on a custom-developed 96-channel electrochemical reader (EZLife Bio, Woodland Hills, CA). The device consists of a high-throughput electrochemical potentiostat system that is able to apply a fixed voltage and perform electrochemical readout on 96 channels simultaneously. A mixture of 2.5 μg/ml recombinant SSA/Ro-52 Ag expressed by recombinant baculovirus infection of *Spodoptera frugiperda* Sf9 insect cells (Surmodics, Eden Prairie, MN), pyrrole (W338605; Sigma-Aldrich Corp.), and 3 mM potassium chloride was diluted in UltraPure water (Thermo Fisher Scientific, Waltham, MA). The mixture was vortexed, and a quantity of 30 μl was loaded onto each electrode on the 96-well gold electrode plate (EZLife Bio). To immobilize the Ag to the surface of the electrode, a cyclic square-wave electrode field was applied for five cycles of 1 s at 350 mV and 1 s of 950 mV (10 s total). After the electrochemical polymerization, each electrode was washed for three cycles in PBS-T buffer (1× PBS [Affymetrix Inc., Sunnyvale, CA] and 0.05% Tween 20 [Bio-Rad Laboratories, Hercules, CA]).

For the SSA/Ro-52 Ab immunoassay, saliva was diluted in a blocker casein solution (1% w/v purified casein, pH 7.4; Thermo Fisher Scientific) at a volume ratio of 1:64. A quantity of 30 μl diluted saliva was loaded onto each electrode coated with capture Ag and then incubated for 30 min. Following incubation, each electrode was washed for three cycles in PBS-T buffer.

https://doi.org/10.4049/immunohorizons.2300043

The secondary Ab, biotinylated polyclonal anti-human IgG (H+L) (Thermo Fisher Scientific) was diluted in blocker casein to a concentration of 2500 ng/ml. A quantity of 30 μl diluted secondary Ab solution was pipetted onto each electrode and then incubated for 30 min. Following incubation, each electrode was washed for three cycles with PBS-T buffer. For the final incubation, Pierce Streptavidin Poly-HRP (Thermo Fisher Scientific) was diluted in blocker casein solution (1:2000), and 30 μl diluted Ab was loaded onto each electrode and incubated for 30 min, followed by three wash cycles, as described in previous steps. Finally, 60 μl 3,3′,5,5′-tetramethylbenzidine substrate solution (Life Technologies, Carlsbad, CA) was pipetted onto each sensor. The current readout was performed by applying a potential of −200 mV for 60 s to each sensor.

The EFIRM assay was performed by an investigator (S.K.) who was blinded with regard to information on patient diagnosis. Each set of experiments was run independently according to a stratified randomization design to ensure that saliva samples from patients with pSS, patients with non-pSS sicca, and healthy control subjects were evenly distributed across the experimental runs. The experiments were run in duplicate, and the final result was obtained by taking the geometric mean across the duplicates.

Standard curve for EFIRM immunoassay

A standard curve was generated using blocker casein solution spiked with human SSA/Ro-52 Abs (Lifespan Biosciences, Seattle, WA).

Statistical analysis

The discriminatory performance of anti-SSA/Ro-52 autoantibodies measured in saliva was assessed using the area under the receiver operating characteristic (ROC) curve (AUC). The associated 95% confidence interval (CI) was constructed using DeLong's method to estimate the variance. The strength of association between salivary anti-SSA/Ro-52 Abs and diagnosis for each pairwise comparison of patients with pSS, patients with non-pSS sicca, and healthy control subjects was measured using the odds ratio and associated 95% CI. The correlation between serum and saliva measurements of SSA/Ro autoantibodies was assessed using Spearman's rank correlation.

To evaluate the discriminatory performance of anti-SSA/Ro-52 autoantibodies between patients with pSS and healthy subjects, we predefined a threshold cutoff for healthy subjects. The threshold was determined as the 95th percentile of measurable anti-SSA/Ro-52 autoantibodies in the saliva of healthy subjects. To ensure that 95% of patients with pSS are correctly identified while ruling out patients with non-pSS sicca syndrome from additional invasive biopsies clinically, we estimated the specificity associated with 95% sensitivity on the ROC curve for the comparison of pSS versus non-pSS sicca. Statistical significance was considered to be achieved if the p value was less than 0.05.

RESULTS

Baseline characteristics of patients

Thirty-four patients with pSS and 35 patients with non-pSS sicca syndrome were enrolled (Table I). The study population was predominantly female, and the mean age (±SD) was 54.6 ± 10.5 y in the pSS group and 57.3 ± 14.5 y in the non-pSS sicca group. The rate of dry mouth or dry eye did not differ between the groups. More patients with pSS had a detectable antinuclear Ab (ANA) titer than did the patients with non-pSS sicca (88.2% versus 34.3%; $p < 0.001$). Serum anti-SSA/Ro and anti-SSB/La autoantibodies (measured by ELISA at diagnostic work-up) were present in 85.3% (29 of 34) and 64.7% (22 of 34) of patients with pSS, respectively, whereas none of the patients with non-pSS sicca had detectable serum anti-SSA/Ro or anti-SSB/La autoantibodies. The histological analysis of the labial salivary gland tissue biopsies from the patients with non-pSS sicca did not reveal the presence of focal lymphocytic infiltrates (i.e., the focus score was below 1). A focus was defined as an aggregate (mostly periductal localization) of at least 50 mononuclear cells. To calculate the focus score, defined as the number of foci per 4 mm^2, the number of foci in the glandular section was divided by the total glandular area and multiplied by 4 (18). A focus score ≥1 was present in 22 (81.5%) of 27 patients with pSS. Keratoconjunctivitis sicca (19), defined as a positive Schirmer's test result (≤5 mm of the strip is wet after 5 min) and/or ocular staining score ≥5 or van Bijsterveld score ≥4 in at least one eye, was more common in the patients with pSS than in the patients with non-pSS sicca (91.2% versus 60.0%; $p = 0.005$) (Table I).

EFIRM immunoassay

The EFIRM immunoassay was developed to detect salivary anti-SSA/Ro-52 autoantibodies using recombinant human SSA/Ro-52 polymerized onto the gold surface of EFIRM electrodes (Fig. 1A). The 52 kDa SSA subunit was chosen as an Ag target to capture salivary SSA/Ro-52 autoantibodies because anti-SSA/Ro-52 has the highest positive predictive value (100%) compared with that of anti-SSA/Ro-60 (25%) in serum (13). The assay for detection of salivary SSA/Ro-52 autoantibodies was optimized using human anti-SSA/Ro-52 to generate an optimal calibration curve. Human anti-SSA/Ro-52 was spiked into unstimulated whole saliva collected from healthy subjects to demonstrate the titratability and optimization of the signal-to-noise ratio (Supplemental Figs. 1 and 2).

Detection of SSA/Ro-52 autoantibody in saliva

We investigated the association between the serum and salivary SSA/Ro autoantibody levels. Igs (isotypes and subtypes) have been found to be linearly distributed between the plasma and saliva (20). EFIRM quantitatively detected salivary anti-SSA/Ro-52 in 77% (53 of 69) of the combined pSS and sicca cohorts, outperforming ELISA, which detected only 42% (29 of 69) seropositivity in the same cohort. Therefore, the serum SSA/Ro autoantibody titers (measured by ELISA) correlated moderately ($r = 0.47$; 95% CI, 0.26–0.65) with salivary anti-SSA/Ro-52 autoantibody levels (measured by EFIRM) in the pSS and non-pSS sicca cohorts (Fig. 1B).

TABLE I. Demographic characteristics of patients with pSS and patients with non-pSS sicca

	pSS (n = 34)	Non-pSS Sicca (n = 35)	p Value
Age, y	54.0 ± 10.5	57.8 ± 14.5	0.207
Female	34/34 (100.0%)	31/35 (88.6%)	0.116
Symptom duration, mo	46.1 ± 53.5	47.0 ± 83.4	0.960
Ocular dryness	28/33 (84.8%)	32/35 (91.4%)	0.471
Oral dryness	29/33 (87.9%)	29/33 (87.9%)	1.000
Unstimulated whole saliva flow rate ≤0.10 ml/min	6/9 (66.7%)	—	—
Labial salivary gland focus score ≥1	22/27 (81.5%)	1/29 (3.4%)	<0.001
Keratoconjunctivitis sicca[a]	31/34 (91.2%)	21/35 (60.0%)	0.005
Positive ANA titer	30/34 (88.2%)	12/35 (34.3%)	<0.001
Positive serum SSA/Ro autoantibody	29/34 (85.3%)	0/35 (0%)	<0.001
Positive serum SSB/La autoantibody	22/34 (64.7%)	0/35 (0%)	<0.001

Values are given as the mean and SD and the number of patients (percent). Statistical significance was considered to be achieved if the p value was <0.05.
[a]Keratoconjunctivitis sicca was defined as a positive Schirmer test result (≤5 mm of strip is wet after 5 min) and/or an ocular staining score ≥5 or van Bijsterveld score ≥4 in at least one eye.

Next, salivary levels of anti-SSA/Ro-52 autoantibodies in the 34 patients with pSS, 35 patients with non-pSS sicca, and 41 healthy subjects were measured (Fig. 2). Salivary anti-SSA/Ro-52 autoantibodies were detected in 94% (32 of 34) patients with pSS, whereas only 85% (29 of 34) exhibited serum positivity. Of the five patients with pSS with anti-SSA/Ro-negative serum, four (80%) displayed EFIRM-measurable SSA/Ro-52 autoantibodies in saliva. Out of 29 patients with pSS with anti-SSA/Ro-positive serum, 28 (97%) had anti-SSA/Ro-52 Abs in their saliva. Surprisingly, 60% (21 of 35) of patients with non-pSS sicca with anti-SSA/Ro-negative serum had EFIRM-detectable SSA/Ro-52 autoantibodies in their saliva. EFIRM detected salivary anti-SSA in 2 of the 41 healthy control subjects.

Salivary SSA/Ro-52 autoantibodies as a biomarker to discriminate patients with pSS, patients with non-pSS sicca, and healthy subjects

We explored the potential of salivary anti-SSA/Ro-52 autoantibodies as a biomarker to differentiate patients with pSS, patients with non-pSS sicca, and healthy control subjects (Fig. 3). The AUC is shown within each panel of Fig. 3: pSS versus non-pSS sicca, 0.75 (95% CI, 0.63–0.86) (Fig. 3A); non-pSS sicca versus control, 0.85 (95% CI, 0.76–0.94) (Fig. 3B); pSS versus control, 0.98 (95% CI, 0.94–1.0) (Fig. 3C); and combined pSS and non-pSS sicca versus control, 0.91 (95% CI, 0.86–0.96) (Fig. 3D). In addition, we estimated the odds ratios for each pairwise comparison of interest: pSS versus non-pSS sicca, 3.63 (95% CI, 1.69–7.81); non-pSS sicca versus healthy, 8.05 (95% CI, 3.24–19.99); pSS versus healthy, 62.41 (95% CI, 8.24–472.57); and combined non-pSS sicca and pSS versus healthy, 11.24 (95% CI, 4.77–26.50). The specificity associated with 95% sensitivity on the ROC curve for the comparison of pSS versus non-pSS sicca was 37.1% (95% CI, 5.7–54.3%).

Factors associated with measurable salivary SSA/Ro-52 autoantibodies in patients with non-pSS sicca

We investigated whether patients with non-pSS sicca with measurable and nonmeasurable saliva anti-SSA/Ro-52 autoantibodies differed in clinical demographics. Subjective symptoms such as dry mouth and dry eyes, as well as objective measures such as positive rheumatoid factor and ocular staining score, did not differ between the groups. Patients with non-pSS sicca who had

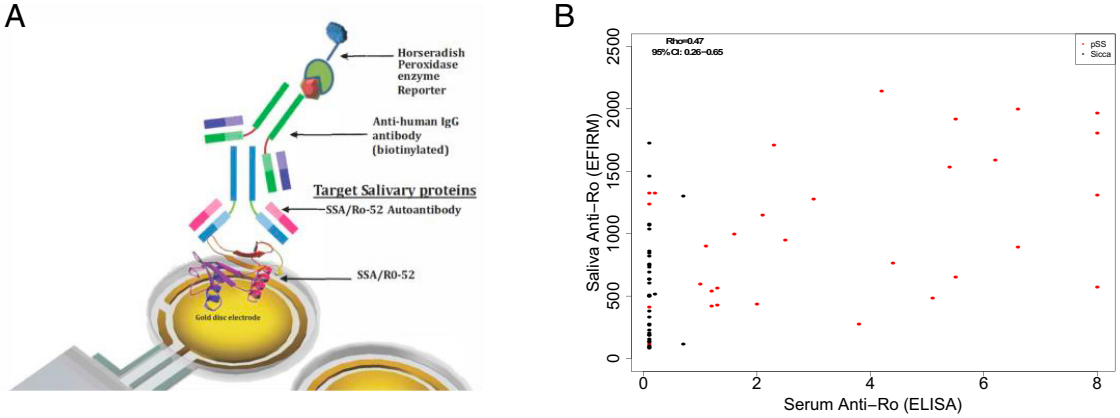

FIGURE 1. (**A**) Schema of the EFIRM SSA/Ro-52 immunoassay. (**B**) Correlation between SSA/Ro autoantibodies in serum (ELISA) and saliva (EFIRM) in patients with pSS or patients with non-pSS sicca using Spearman correlation analysis. Serum Abs are expressed as titers (mg/dl) and saliva Abs as current (nAmp).

https://doi.org/10.4049/immunohorizons.2300043

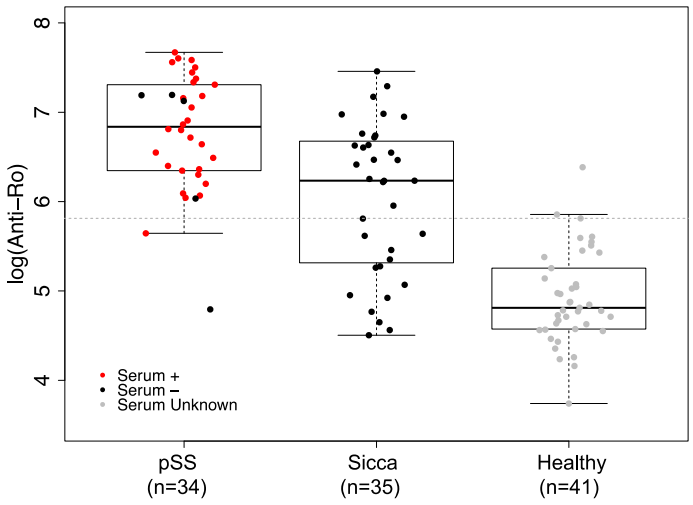

FIGURE 2. EFIRM detection of SSA/Ro autoantibodies in saliva of patients with pSS, patients with non-pSS sicca, and healthy control subjects.

The dashed line indicates the threshold for measurability of salivary anti-SSA/Ro-52. Serum positive results are indicated by red dots, serum negative results are indicated by black dots, and unknown serum status is indicated by gray dots.

measurable salivary anti-SSA/Ro-52 autoantibodies were younger (53.7 versus 63.6 y; $p = 0.033$) and had higher rates of positive ANA than those with nonmeasurable anti-SSA/Ro-52 autoantibodies in their saliva (42.9% versus 21.4%; $p = 0.282$) (Table II).

DISCUSSION

The diagnosis of pSS is complex. Clinical and laboratory features of the pSS and non-pSS sicca study cohorts (Table I) share characteristics similar to those of previously reported patient cohorts (8, 21) and fulfill the most recent 2016 ACR/European League Against Rheumatism diagnostic criteria of pSS. The criteria primarily rely on serology (presence of SSA/Ro Ab) and labial salivary gland biopsy (with a focus score ≥ 1) (22). However, it is likely that a number of patients who do not meet the ACR/European League Against Rheumatism criteria remain undiagnosed (23) because of poor serum-based immunoprecipitation assay and ELISA sensitivity of 61% (35 of 57), 67% (18 of 27), and 71% (47 of 66) in serum anti-SSA/Ro-52 detection (10, 24, 25). It has been shown that at least one circulatory autoantibody (i.e., ANA, rheumatoid factor, anti-SSA/Ro-60, anti-SSA/Ro-52, or anti-SSB/La) is present in patients with pSS as early as 19 to 20 y (median, 4.3–5.1 y) before the diagnosis (13). Because preventive measures are important in both the pSS and non-pSS sicca groups, early and accurate diagnosis is important.

The absence of circulating anti-SSA/Ro autoantibodies and a negative labial salivary gland biopsy do not exclude the possibility of an ongoing localized autoimmune process in the exocrine glands. Histopathological challenges such as smoking and corticosteroids can influence the presence of focal lymphocytic infiltrates and can reverse histopathological presentations, respectively (26, 27). Simple noninvasive alternatives in detecting anti-SSA/Ro autoantibodies in saliva can serve as an additional screening test for early clinical diagnosis of autoimmunity and before the occurrence of multiorgan involvement and organ damage. Previous findings indicate that anti-SSA/Ro and anti-SSB/La Abs are produced locally by the lymphocytes in the salivary glands of patients with pSS (9, 28). In one study, the anti-SSA/Ro and anti-SSB/La Ab-producing cells contained Abs of predominantly the IgA isotype (28), and in another study, the labial salivary gland lymphocytes produced Abs against bovine Ro 60 kDa of the IgG isotype and a minor fraction of the IgA and IgM isotypes (9). Our findings support these findings of a local synthesis of autoantibodies in the salivary gland tissue, even before the detection of these autoantibodies in serum. Furthermore, these Abs may be more Ag-specific IgA Abs than IgG Abs. Halse et al. (9) suggested that salivary enrichment of Ag-specific IgA and IgM can be explained by an active transepithelial transport of these isotypes, which can be obtained through an interplay between the mucosal B cells and the transmembrane secretory component, which is the receptor responsible for external transport of locally produced polymeric IgA and IgM (9). We are planning to address different Ig isotypes in future studies.

Because autoantibodies can be produced locally by the infiltrating lymphocytes in glandular tissue and consequently secreted in saliva before their appearance in serum, detection of SSA/Ro-52 autoantibodies in saliva might assist in earlier detection of autoimmunity and replace serum anti-SSA/Ro Ab testing in the diagnosis and monitoring of pSS. Previous studies did not find any discriminatory value of using salivary SSA/Ro autoantibodies for pSS diagnostics, and they were unable to quantify the salivary autoantibodies (11, 12). To our knowledge, this study is the first saliva immunoassay with clinical sensitivity to quantitatively detect anti-SSA/Ro-52 autoantibodies in the saliva of 80% (4 of 5) of patients with pSS and 60% (21 of 35) of patients with non-pSS sicca, who were seronegative at the time the diagnostic work-up was performed. This is in contrast to previous studies that detected salivary anti-SSA only in seropositive patients with Sjögren's syndrome (8–10).

Interestingly, 60% of the patients with non-pSS sicca had salivary anti-SSA/Ro autoantibodies in their saliva. These findings may indicate early onset of pSS. A future study should entail a prospective cohort to determine if these patients with sicca symptoms and anti-SSA/Ro autoantibodies in their saliva develop manifest pSS. This will be important for therapeutic intervention early in the disease course and before irreversible immune-mediated destruction of the salivary (exocrine) glands.

Salivary anti-SSA/Ro-52 autoantibodies measured by EFIRM discriminated patients with pSS or non-pSS sicca from healthy subjects, with an AUC of 0.91 (95% CI, 0.86–0.96) and an odds ratio of 11.24 (95% CI, 4.77–26.5). In addition, the odds of presenting with anti-SSA/Ro-52 autoantibodies in saliva are 62 times higher in pSS or 8 times higher in patients with sicca than in healthy subjects, with AUCs of 0.98 and 0.85, respectively. In the present study, we demonstrated the utility of the proposed

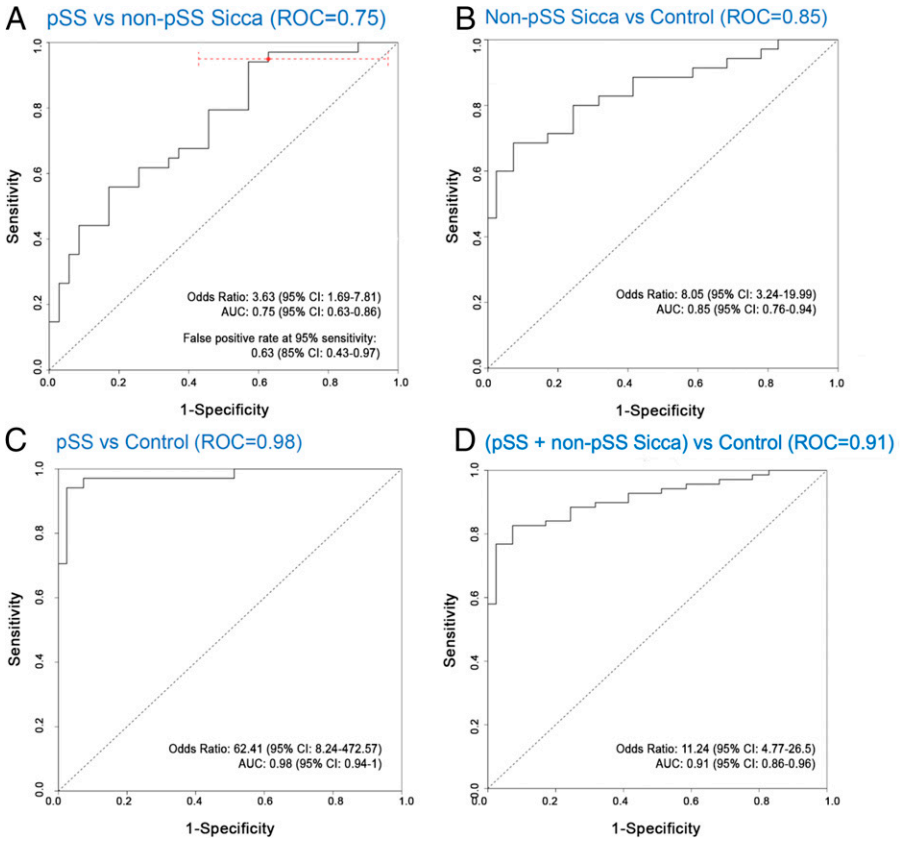

FIGURE 3. Performance of salivary SSA/Ro-52 autoantibody EFIRM immunoassay.

The AUC with 95% CI was used to discriminate patients with pSS, patients with non-pSS sicca, and healthy control subjects. (**A**) AUC of pSS versus non-pSS sicca. (**B**) AUC of non-pSS sicca versus control. (**C**) AUC of pSS versus control. (**D**) AUC of combined pSS/non-pSS sicca versus control.

saliva-based immunoassay in SSA/Ro-52 autoantibody detection to fast-track autoimmune diagnosis. Previous studies indicated the absence of circulatory SSA/Ro-52 autoantibodies in patients with salivary gland hypofunction due to radiation therapy to the head and neck region, intake of xerogenic medication, HIV, hepatitis C virus, sarcoidosis, amyloidosis, graft-versus-host disease, and IgG4-related disease (29–33). Future experiments can be carried out for salivary SSA/Ro-52 autoantibodies in these cohorts for additional comparisons.

The limitation of this study is that it included the detection and quantification of only anti-SSA/Ro-52 salivary autoantibodies. SSA/Ro autoantibodies react against two different SSA Ags: Ro-52 (a 52 kDa protein) and Ro-60 (a 60 kDa protein). On the one hand, the 60 kDa target Ag for SSA/Ro-60 autoantibodies is a ribonucleoprotein complex containing a small cytoplasmic RNA (34). On the other hand, Ro-52 is an IFN-induced protein of the tripartite motif family that was initially described as a part of the SSA/Ro ribonucleoprotein complex but is now considered a separate Ag that can exist both with and without the presence of Ro-60 (25, 34). Future studies will include a saliva EFIRM immunoassay for the 60 kDa SSA/Ro subunit. The combination of Ro-52 and Ro-60 may increase diagnostic accuracy for longitudinal investigations.

TABLE II. Clinical characteristics of patients with non-pSS sicca with measurable and nonmeasurable SSA/Ro-52 autoantibody in saliva

	Measurable (n = 21)	Nonmeasurable (n = 14)	p Value
Age, y	53.7 ± 15.3	63.6 ± 11.4	0.033
Female	18/21 (85.7%)	13/14 (92.9%)	0.626
Symptom duration, mo	36.7 ± 32.5	61.3 ± 124.6	0.499
Ocular dryness	20/21 (95.2%)	12/14 (85.7%)	0.551
Oral dryness	17/20 (85.0%)	12/13 (92.3%)	1.000
Labial salivary gland focus score ≥1	0/16 (0%)	1/13 (7.7%)	0.448
Keratoconjunctivitis sicca[a]	13/20 (65.0%)	8/13 (61.5%)	1.000
Positive ANA titer	9/21 (42.9%)	3/14 (21.4%)	0.282
Positive serum SSA/Ro autoantibody	0/21 (0%)	0/14 (0%)	—
Positive serum SSB/La autoantibody	0/21 (0%)	0/14 (0%)	—

Values are given as the mean and SD and the number of patients (percent). Statistical significance was considered to be achieved if the p value was <0.05.
[a]Keratoconjunctivitis sicca was defined as a positive Schirmer test result (≤5 mm of strip is wet after 5 min) and/or an ocular staining score ≥5 or van Bijsterveld score ≥4 in at least one eye.

https://doi.org/10.4049/immunohorizons.2300043

The EFIRM platform, which, to our knowledge, is a novel development, addresses an unmet clinical need by noninvasively quantifying saliva anti-SSA/Ro-52, permitting early detection in seronegative patients with pSS and patients with non-pSS sicca. The saliva anti-SSA/Ro-52 immunoassay can impactfully serve as a screening test to distinguish patients with pSS from patients with non-pSS sicca and healthy subjects. Improving diagnoses in the heterogeneous clinical presentation of pSS will permit timely therapeutic intervention early in the disease course.

DISCLOSURES

D.T.W.W. is consultant to GlaxoSmithKline, Mars Wrigley, and Colgate-Palmolive and has ownership in Liquid Diagnostics LLC and RNAmeTRIX. No other disclosures relevant to this article were reported. The other authors have no financial conflicts of interest.

REFERENCES

1. Kassan, S. S., and H. M. Moutsopoulos. 2004. Clinical manifestations and early diagnosis of Sjögren syndrome. *Arch. Intern. Med.* 164: 1275–1284.
2. Ramos-Casals, M., A. G. Tzioufas, and J. Font. 2005. Primary Sjögren's syndrome: new clinical and therapeutic concepts. *Ann. Rheum. Dis.* 64: 347–354.
3. Mariette, X., and L. A. Criswell. 2018. Primary Sjögren's syndrome. *N. Engl. J. Med.* 378: 931–939.
4. Brito-Zerón, P., N. Acar-Denizli, M. Zeher, A. Rasmussen, R. Seror, E. Theander, X. Li, C. Baldini, J. E. Gottenberg, D. Danda, et al.; EULAR-SS Task Force Big Data Consortium. 2017. Influence of geolocation and ethnicity on the phenotypic expression of primary Sjögren's syndrome at diagnosis in 8310 patients: a cross-sectional study from the Big Data Sjögren Project Consortium. *Ann. Rheum. Dis.* 76: 1042–1050.
5. Villa, A., A. Wolff, N. Narayana, C. Dawes, D. J. Aframian, A. M. Lynge Pedersen, A. Vissink, A. Aliko, Y. W. Sia, R. K. Joshi, et al. 2016. World Workshop on Oral Medicine VI: a systematic review of medication-induced salivary gland dysfunction. *Oral Dis.* 22: 365–382.
6. Manoussakis, M. N., and E. K. Kapsogeorgou. 2010. The role of intrinsic epithelial activation in the pathogenesis of Sjögren's syndrome. *J. Autoimmun.* 35: 219–224.
7. Alunno, A., F. Carubbi, O. Bistoni, S. Caterbi, E. Bartoloni, G. Mirabelli, F. Cannarile, P. Cipriani, R. Giacomelli, and R. Gerli. 2015. T regulatory and T helper 17 cells in primary Sjögren's syndrome: facts and perspectives. *Mediators Inflamm.* 2015: 243723.
8. Ben-Eli, H., A. Solomon, D. J. Aframian, E. Ben-Chetrit, D. Mevorach, G. Kleinstern, T. Waterboer, M. Willhauck-Fleckenstein, M. Pawlita, and O. Paltiel. 2020. Serological and hematological characteristics of Sjogren's syndrome and dry eye syndrome patients using a novel immune serology technique. *PLoS One* 15: e0244712.
9. Halse, A., J. B. Harley, U. Kroneld, and R. Jonsson. 1999. Ro/SS-A-reactive B lymphocytes in salivary glands and peripheral blood of patients with Sjögren's syndrome. *Clin. Exp. Immunol.* 115: 203–207.
10. Ching, K. H., P. D. Burbelo, M. Gonzalez-Begne, M. E. P. Roberts, A. Coca, I. Sanz, and M. J. Iadarola. 2011. Salivary anti-Ro60 and anti-Ro52 antibody profiles to diagnose Sjogren's syndrome. *J. Dent. Res.* 90: 445–449.
11. Horsfall, A. C., L. M. Rose, and R. N. Maini. 1989. Autoantibody synthesis in salivary glands of Sjögren's syndrome patients. *J. Autoimmun.* 2: 559–568.
12. Wei, P., C. Li, L. Qiang, J. He, Z. Li, and H. Hua. 2015. Role of salivary anti-SSA/B antibodies for diagnosing primary Sjögren's syndrome. *Med. Oral Patol. Oral Cir. Bucal* 20: e156–e160.
13. Theander, E., R. Jonsson, B. Sjöström, K. Brokstad, P. Olsson, and G. Henriksson. 2015. Prediction of Sjögren's syndrome years before diagnosis and identification of patients with early onset and severe disease course by autoantibody profiling. *Arthritis Rheumatol.* 67: 2427–2436.
14. Chiang, S. H., M. Tu, J. Cheng, F. Wei, F. Li, D. Chia, O. Garner, S. Chandrasekaran, R. Bender, C. M. Strom, and D. T. W. Wong. 2021. Development and validation of a quantitative, non-invasive, highly sensitive and specific, electrochemical assay for anti-SARS-CoV-2 IgG antibodies in saliva. *PLoS One* 16: e0251342.
15. Shiboski, S. C., C. H. Shiboski, L. Criswell, A. Baer, S. Challacombe, H. Lanfranchi, M. Schiødt, H. Umehara, F. Vivino, Y. Zhao, et al. 2012. American College of Rheumatology classification criteria for Sjögren's syndrome: a data-driven, expert consensus approach in the SICCA cohort. *Arthritis Care Res. (Hoboken)* 64: 475–487.
16. Pedersen, A. M., J. Reibel, H. Nordgarden, H. O. Bergem, J. L. Jensen, and B. Nauntofte. 1999. Primary Sjögren's syndrome: salivary gland function and clinical oral findings. *Oral Dis.* 5: 128–138.
17. Hu, S., J. Wang, J. Meijer, S. Ieong, Y. Xie, T. Yu, H. Zhou, S. Henry, A. Vissink, J. Pijpe, et al. 2007. Salivary proteomic and genomic biomarkers for primary Sjögren's syndrome. *Arthritis Rheum.* 56: 3588–3600.
18. Fisher, B. A., R. Jonsson, T. Daniels, M. Bombardieri, R. M. Brown, P. Morgan, S. Bombardieri, W. F. Ng, A. G. Tzioufas, C. Vitali, et al.; Sjögren's histopathology workshop group (appendix) from ESSENTIAL (EULAR Sjögren's syndrome study group). 2017. Standardisation of labial salivary gland histopathology in clinical trials in primary Sjögren's syndrome. *Ann. Rheum. Dis.* 76: 1161–1168.
19. Whitcher, J. P., C. H. Shiboski, S. C. Shiboski, A. M. Heidenreich, K. Kitagawa, S. Zhang, S. Hamann, G. Larkin, N. A. McNamara, J. S. Greenspan, and T. E. Daniels; Sjögren's International Collaborative Clinical Alliance Research Groups. 2010. A simplified quantitative method for assessing keratoconjunctivitis sicca from the Sjögren's Syndrome International Registry. *Am. J. Ophthalmol.* 149: 405–415.
20. Yan, W., R. Apweiler, B. M. Balgley, P. Boontheung, J. L. Bundy, B. J. Cargile, S. Cole, X. Fang, M. Gonzalez-Begne, T. J. Griffin, et al. 2009. Systematic comparison of the human saliva and plasma proteomes. *Proteomics Clin. Appl.* 3: 116–134.
21. Goules, A. V., O. D. Argyropoulou, V. C. Pezoulas, L. Chatzis, E. Critselis, S. Gandolfo, F. Ferro, M. Binutti, V. Donati, S. Zandonella Callegher, et al. 2020. Primary Sjögren's syndrome of early and late onset: distinct clinical phenotypes and lymphoma development. *Front. Immunol.* 11: 594096.
22. Shiboski, C. H., S. C. Shiboski, R. Seror, L. A. Criswell, M. Labetoulle, T. M. Lietman, A. Rasmussen, H. Scofield, C. Vitali, S. J. Bowman, and X. Mariette. 2017. 2016 ACR-EULAR classification criteria for primary Sjögren's syndrome: a consensus and data-driven methodology involving three international patient cohorts. *Arthritis Rheumatol.* 69: 35–45.
23. Narváez, J., S. A. Sánchez-Fernández, D. Seoane-Mato, F. Díaz-González, and S. Bustabad. 2020. Prevalence of Sjögren's syndrome in the general adult population in Spain: estimating the proportion of undiagnosed cases. *Sci. Rep.* 10: 10627.
24. Burbelo, P. D., H. P. Leahy, A. T. Issa, S. Groot, J. N. Baraniuk, N. P. Nikolov, G. G. Illei, and M. J. Iadarola. 2009. Sensitive and robust luminescent profiling of anti-La and other autoantibodies in Sjogren's syndrome. *Autoimmunity* 42: 515–524.
25. Infantino, M., F. Meacci, V. Grossi, M. Benucci, G. Morozzi, E. Tonutti, M. Tampoia, A. Ott, W. Meyer, F. Atzeni, et al. 2015. Serological epitope profile of anti-Ro52-positive patients with systemic autoimmune rheumatic diseases. *Arthritis Res. Ther.* 17: 365.

26. Zandbelt, M. M., F. H. van den Hoogen, P. C. de Wilde, P. J. van den Berg, H. G. Schneider, and L. B. van de Putte. 2001. Reversibility of histological and immunohistological abnormalities in sublabial salivary gland biopsy specimens following treatment with corticosteroids in Sjögren's syndrome. *Ann. Rheum. Dis.* 60: 511–513.
27. Stone, D. U., D. Fife, M. Brown, K. E. Earley, L. Radfar, C. E. Kaufman, D. M. Lewis, N. L. Rhodus, B. M. Segal, D. J. Wallace, et al. 2017. Effect of tobacco smoking on the clinical, histopathological, and serological manifestations of Sjögren's syndrome. *PLoS One* 12: e0170249.
28. Tengnér, P., A. K. Halse, H. J. Haga, R. Jonsson, and M. Wahren-Herlenius. 1998. Detection of anti-Ro/SSA and anti-La/SSB autoantibody-producing cells in salivary glands from patients with Sjögren's syndrome. *Arthritis Rheum.* 41: 2238–2248.
29. Schiødt, M., D. Greenspan, T. E. Daniels, J. Nelson, P. J. Leggott, D. W. Wara, and J. S. Greenspan. 1989. Parotid gland enlargement and xerostomia associated with labial sialadenitis in HIV-infected patients. *J. Autoimmun.* 2: 415–425.
30. Mayo, M. J. 2003. Extrahepatic manifestations of hepatitis C infection. *Am. J. Med. Sci.* 325: 135–148.
31. Palekar, N. A., and S. A. Harrison. 2005. Extrahepatic manifestations of hepatitis C. *South. Med. J.* 98: 1019–1023.
32. Alavian, S. M., N. Mahboobi, N. Mahboobi, and P. Karayiannis. 2013. Oral conditions associated with hepatitis C virus infection. *Saudi J. Gastroenterol.* 19: 245–251.
33. Mortazavi, H., M. Baharvand, A. Movahhedian, M. Mohammadi, and A. Khodadoustan. 2014. Xerostomia due to systemic disease: a review of 20 conditions and mechanisms. *Ann. Med. Health Sci. Res.* 4: 503–510.
34. Yoshimi, R., A. Ueda, K. Ozato, and Y. Ishigatsubo. 2012. Clinical and pathological roles of Ro/SSA autoantibody system. *Clin. Dev. Immunol.* 2012: 606195.