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ORIGINAL ARTICLE

# Preclinical Validation of Salivary Biomarkers for Primary Sjögren's Syndrome

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***Objective.*** **Sjögren's syndrome (SS) is a systemic autoimmune disease with a variety of presenting symptoms that may delay its diagnosis. We previously discovered a number of candidate salivary biomarkers for primary SS using both mass spectrometry and expression microarray analysis. In the current study, we aimed to verify these candidate biomarkers in independent patient populations and to evaluate their predictive values for primary SS detection.**

***Methods.*** **In total, 34 patients with primary SS, 34 patients with systemic lupus erythematosus (SLE), and 34 healthy individuals were enrolled for the validation studies. Salivary protein biomarkers were measured using either Western blotting or enzyme-linked immunosorbent assay, and the messenger RNA (mRNA) biomarkers were measured using quantitative polymerase chain reaction. Statistical analysis was performed using R software, version 2.9.**

***Results.*** **Three protein biomarkers (cathepsin D [CPD], $\alpha$-enolase, and $\beta_2$-microglobulin [$\beta_2$m]) and 3 mRNA biomarkers (myeloid cell nuclear differentiation antigen [MNDA], guanylate binding protein 2 [GBP-2], and low-affinity IIIb receptor for the Fc fragment of IgG) were significantly elevated in patients with primary SS compared with both SLE patients and healthy controls. The combination of 3 protein biomarkers, CPD, $\alpha$-enolase, and $\beta_2$m, yielded a receiver operating characteristic (ROC) value of 0.99 in distinguishing primary SS from healthy controls. The combination of protein biomarkers $\beta_2$m and 2 mRNA biomarkers, MNDA and GBP-2, reached an ROC of 0.95 in discriminating primary SS from SLE.**

***Conclusion.*** **We have successfully verified a panel of protein and mRNA biomarkers that can discriminate primary SS from both SLE and healthy controls. If further validated in patients with primary SS and those with sicca symptoms but no autoimmune disease, these biomarkers may lead to a simple yet highly discriminatory clinical tool for diagnosis of primary SS.**

## INTRODUCTION

Sjögren's syndrome (SS) is a common autoimmune disease, with an estimated prevalence of 1–4 million patients in the US (1). The syndrome is characterized by progressive inflammation of the exocrine glands, in particular the salivary and lacrimal glands, frequently in combination with extraglandular manifestations. Histopathologically, expression of HLA–DR in glandular epithelial cells, lymphocytic infiltration of glandular tissue, and sustained localized cytokine production are present (2). Consequently, patients with SS experience irreversible damage of salivary and lacrimal glands and loss of saliva and tear production (dry mouth and eyes). SS primarily affects women, with a ratio of 9:1 over the occurrence in men. The disease may occur alone as primary SS or present as secondary SS when it is associated with other autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus (SLE). Regarding treatment of SS, a gain in knowledge regarding the immunopathogenesis has resulted in new strategies for therapeutic intervention, in particular with the B lymphocyte–depleting drug rituximab (3). Rituximab is a chimeric monoclonal antibody against the B cell surface antigen CD20. Initial clinical trials suggested that rituximab is an effective agent in the treatment of SS and mucosa-associated lymphoid tissue–type lymphoma (4–10).

Diagnosing SS is complicated by the variety of presenting symptoms a patient may manifest, and the similarity between some symptoms from SS and those caused by other autoimmune disorders. In 2002, an international group reached consensus on a set of US/European criteria for SS classification (11). Classification of primary SS re-

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**Table 1. List of candidate messenger RNA biomarkers**

| Gene name | Full name |
|---|---|
| EGR-1 | Early growth response 1 |
| $\beta_2$m | $\beta_2$-microglobulin |
| BTG2 | B cell translocation gene family, member 2 |
| GBP-2 | Guanylate binding protein 2, interferon-inducible |
| MNDA | Myeloid cell nuclear differentiation antigen |
| FCGR3B | Low-affinity IIIb receptor for the Fc fragment of IgG |
| TXNIP | Thioredoxin interacting protein |
| HLA–B | Major histocompatibility complex, class I, B |

quires 4 of 6 criteria, including a positive minor salivary gland biopsy or antibody to SSA/SSB. Blood tests can determine whether a patient has high levels of anti-Ro/SSA and anti-La/SSB. Anti-La/SSB is more specific but its sensitivity is lower; anti-Ro/SSA is more sensitive but associated with various other autoimmune conditions (12). Classification of secondary SS requires an established connective tissue disease and 1 sicca symptom plus 2 objective tests for dry mouth and eyes at the time of presentation. A minor salivary gland or parotid gland biopsy can reveal lymphocytes clustered around salivary glands and damage to these glands due to inflammation, and therefore this is a specific diagnostic approach for SS. However, the procedure is invasive, is time consuming, requires the evaluation from an expert histopathologist, and may have invertible sequelae (13).

By using mass spectrometry and expression microarray analysis, we previously discovered a set of saliva protein and messenger RNA (mRNA) biomarkers that are potentially valuable for primary SS detection (14). The purpose of the current study was to validate the discovered putative biomarkers in independent patient populations using immunoassays and quantitative real-time polymerase chain reaction (PCR) and to evaluate their predictive values for primary SS. Three proteins and 3 mRNA biomarkers were successfully verified, which may collectively provide a clinical approach for sensitive and specific detection of primary SS.

## MATERIALS AND METHODS

**Patient and control cohorts.** In total, 34 subjects with primary SS, 34 subjects with SLE, and 34 healthy control subjects were recruited at the University Medical Center Groningen, The Netherlands, for this study. The 3 study groups (primary SS, SLE, and healthy control) were well matched for age, sex, and ethnicity. All of the enrolled subjects were white women because primary SS primarily affects women. The mean ± SD age was 47 ± 15 years in the patients with primary SS (n = 34), 46 ± 15 years in the patients with SLE (n = 34), and 41 ± 9 years in the healthy control subjects (n = 34). Both the University of California, Los Angeles and University Medical Center Groningen Institutional Review Board committees had approved the use of clinical samples for this project. All patients and controls had given informed consent. The information pertaining to the human samples was recorded in a manner such that the subjects could not be identified, directly or through identifiers linked to the subjects. All of the patients with primary SS were diagnosed in strict accordance with the American–European Consensus Group criteria for SS, which are commonly used by clinicians to diagnose SS in daily practice. All of the patients with SLE were diagnosed in agreement with the current American College of Rheumatology clinical criteria for this disease (15). Healthy controls used no medication, experienced neither oral nor ocular dryness, and did not have a history of salivary gland pathology. The demographics of the patients and healthy controls are shown in Supplemental Table A (available in the online version of this article at http://www3.interscience.wiley.com/journal/77005015/home). Patients with SS were characterized as early stage (<4 years) or late stage (>4 years) based on the disease duration since their diagnosis (16).

**Sample collection.** Paraffin-stimulated whole saliva samples were collected from patients with primary SS, patients with SLE, and control subjects for comparative analysis. Saliva sample collection was performed at the University Medical Center Groningen using standardized saliva collection protocols (14). After collection, the saliva samples were immediately processed by centrifugation at 2,600 *g* for 15 minutes at 4°C. The supernatant was removed from the pellet and separated immediately for protein or mRNA stabilization and stored at −80°C.

**Immunoassays.** Enzyme-linked immunosorbent assay (ELISA; Genway) was used to determine the level of $\beta_2$-microglobulin ($\beta_2$m) in saliva samples from primary SS, SLE, and control subjects (n = 34 for each group). We used 20 $\mu$l of saliva sample from each subject and diluted it 10 times for ELISA (100 $\mu$l for each well) according to the manufacturer's instruction manual. Samples were analyzed in duplicate, and the protein levels were determined according to the calibration curves established from standards.

Western blotting was performed on the same set of saliva samples (n = 34 for each of the 3 groups) to measure levels of cathepsin D (CPD) and $\alpha$-enolase. Proteins (20 $\mu$l of each saliva sample) were separated on 12% NuPAGE gels (Invitrogen) at 150V and then transferred to a polyvinylidene difluoride membrane using an Invitrogen blot transfer cell. After saturating with 5% milk in Tris buffered saline–Tween buffer (overnight at 4°C), the blots were sequentially incubated for 2 hours at room temperature with primary antibodies and then with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (GE Healthcare). The bands were detected by enhanced chemiluminescence (Amersham) and quantified using Quantity One software (Bio-Rad).

**Automatic RNA extraction from human saliva.** Salivary RNA from 26 healthy controls, 25 subjects with primary SS, and 26 subjects with SLE was isolated. We were unable to extract a large enough amount of total mRNA from the rest of the cases for validation of mRNA biomar-

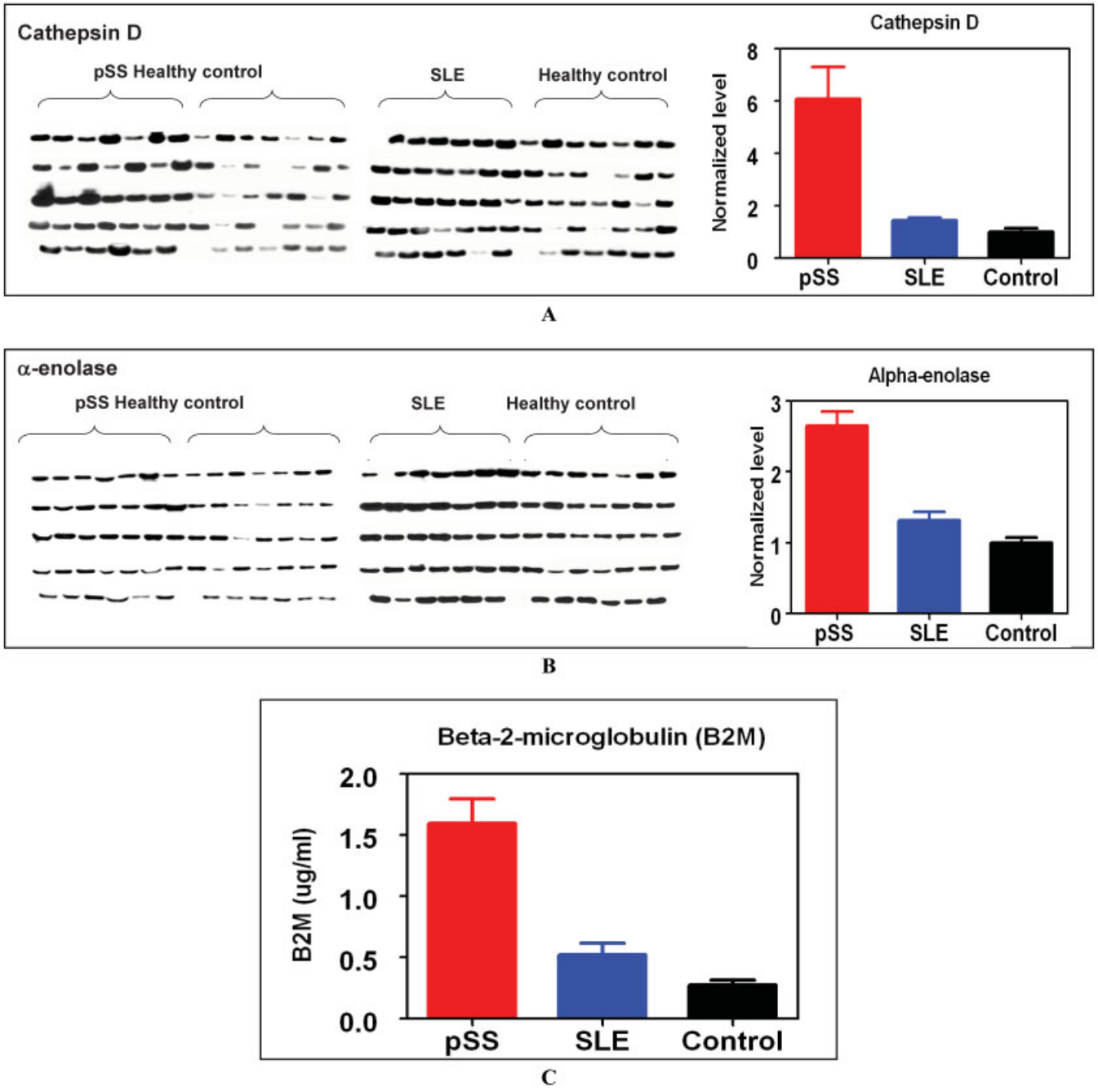


**Figure 1.** Validation of protein biomarkers **A,** cathepsin D (CPD), **B,** α-enolase, and **C,** $\beta_2$-microglobulin ($\beta_2$m) in independent patient (primary Sjögren's syndrome [pSS]) and control (systemic lupus erythematosus [SLE] and healthy control) populations (n = 34 for each group). $\beta_2$m was validated by enzyme-linked immunosorbent assay and mean ± SEM is plotted. CPD and α-enolase were validated by Western blotting, and the bar figures indicate the normalized levels of CPD and α-enolase among 3 groups (mean ± SEM).

kers. For each sample, 330 μL of saliva supernatant was extracted with the MagMax Viral RNA Isolation Kit (Ambion). This process was performed automatically using KingFisher mL technology (Thermo Fisher Scientific), followed by using TURBO DNase treatment (Ambion) to eliminate DNA contamination.

**Real-time quantitative PCR.** Eight candidate mRNA biomarkers were tested in patient and control samples with real-time quantitative PCR (Table 1). These candidates were previously discovered using microarray profiling and preliminarily verified on the same set of samples (n = 10 for primary SS and n = 10 for controls). All primers used for quantitative PCR were designed with the Primer3 program (Whitehead Institute for Biomedical Research; online at http://www.genome.wi.mit.edu) and synthesized by Sigma. Amplicon lengths were approximately 100–130 bp for the outer primer pairs used in preamplification and 60–80 bp for the inner primer pairs used in quantitative PCR analyses (17). After the salivary RNA was reverse-

**Table 2. Performance characteristics of salivary biomarkers for distinguishing primary SS and healthy controls***

| Biomarker | *P*, Wilcoxon | *P*, *t*-test | ROC | Sensitivity | Specificity |
|---|---|---|---|---|---|
| Protein | | | | | |
| $\beta_2$m | $1.25 \times 10^{-10}$ | $1.87 \times 10^{-7}$ | 0.95 | 0.94 | 0.85 |
| Cathepsin D | $5.33 \times 10^{-8}$ | $2.02 \times 10^{-4}$ | 0.88 | 0.76 | 0.88 |
| $\alpha$-enolase | $5.60 \times 10^{-5}$ | $5.76 \times 10^{-5}$ | 0.78 | 0.71 | 0.79 |
| mRNA | | | | | |
| MNDA | $1.49 \times 10^{-5}$ | $5.01 \times 10^{-4}$ | 0.84 | 0.88 | 0.77 |
| FCGR3B | $5.88 \times 10^{-5}$ | $7.19 \times 10^{-3}$ | 0.82 | 0.8 | 0.81 |
| GBP-2 | $3.47 \times 10^{-3}$ | $6.52 \times 10^{-3}$ | 0.74 | 0.76 | 0.69 |
| $\beta_2$m + cathepsin D | | | 0.99 | 1 | 0.92 |
| $\alpha$-enolase + $\beta_2$m + cathepsin D | | | 0.99 | 0.94 | 0.97 |
| MNDA + FCGR3B + GBP-2 | | | 0.86 | 0.88 | 0.81 |

* SS = Sjögren's syndrome; ROC = receiver operating characteristic; mRNA = messenger RNA. See Table 1 for additional definitions.

transcribed using reverse transcriptase and the specific outer primers, quantitative PCR was carried out in reaction volumes of 10 $\mu$l using the SYBR-Green Master Mix (Applied Biosystems) for 15 minutes at 95°C for initial denaturing, followed by 40 cycles of 95°C for 30 seconds and 60°C for 30 seconds with the ABI 7900HT Fast Real Time PCR system (Applied Biosystems). The specificity of the PCR was confirmed according to the melting curve of each gene and the difference in threshold cycle ($\triangle C_t$). The relative expression of each gene was calculated by the comparative $C_t$ method, and the $\triangle C_t$ of each biomarker was used for further analysis (14).

**Statistical analysis.** Statistical software R (R Foundation) was used for computing *P* values and the receiver operating characteristic (ROC) analysis. The plot area under the curve (AUC) was computed via numerical integration of the ROC curves. The optimal cutoff point was determined to yield the maximum corresponding sensitivity and specificity. For the mRNA biomarkers, the $\triangle C_t$ was converted by logistic regression and then the combination ROC of those biomarkers was calculated (18). The biomarker that had the highest AUC value was identified as having the strongest predictive power for detecting primary SS. For the combination of protein and mRNA biomarkers, the ROC analysis was performed on the data obtained from 25 subjects with primary SS, 26 subjects with SLE, and 26 healthy control subjects.

## RESULTS

Three protein biomarkers, CPD, $\alpha$-enolase, and $\beta_2$m, were measured in an independent patient/control cohort (34 subjects with primary SS, 34 subjects with SLE, and 34 healthy controls) to assess their values for primary SS detection. These protein biomarkers were previously identified by using 2-dimensional gel electrophoresis mass spectrometry for a comparative analysis of whole saliva samples from primary SS and healthy control subjects (14). The levels of CPD, $\alpha$-enolase, and $\beta_2$m among the 3 groups, measured by immunoblotting or ELISA, are presented in Figure 1. Statistical analysis suggests that these proteins are significantly overexpressed in primary SS compared with both SLE and healthy control groups (Tables 2 and 3). This panel of protein biomarkers could distinguish patients with primary SS from healthy control individuals with an ROC of 0.99, a sensitivity of 94%, and a specificity of 97% (n = 34 for each group). When used for distinguishing primary SS from the SLE group, the 3 biomarkers reached an ROC of 0.94, a sensitivity of 92%, and a specificity of 88%.

**Table 3. Performance characteristics of salivary biomarkers for distinguishing primary SS and SLE***

| Biomarker | *P*, Wilcoxon | *P*, *t*-test | ROC | Sensitivity | Specificity |
|---|---|---|---|---|---|
| Protein | | | | | |
| $\beta_2$m | $1.54 \times 10^{-8}$ | $1.53 \times 10^{-5}$ | 0.87 | 0.82 | 0.82 |
| Cathepsin D | $2.54 \times 10^{-6}$ | $5.66 \times 10^{-4}$ | 0.82 | 0.76 | 0.88 |
| $\alpha$-enolase | $6.11 \times 10^{-2}$ | $6.96 \times 10^{-2}$ | 0.63 | 0.82 | 0.5 |
| mRNA | | | | | |
| MNDA | $2.23 \times 10^{-4}$ | $2.34 \times 10^{-4}$ | 0.79 | 0.92 | 0.65 |
| FCGR3B | $1.23 \times 10^{-2}$ | $2.49 \times 10^{-3}$ | 0.7 | 0.92 | 0.65 |
| GBP-2 | $6.11 \times 10^{-3}$ | $1.76 \times 10^{-3}$ | 0.72 | 0.8 | 0.73 |
| MNDA + $\beta_2$m + GBP-2 | | | 0.95 | 0.92 | 0.89 |
| $\alpha$-enolase + $\beta_2$m + cathepsin D | | | 0.94 | 0.92 | 0.88 |
| MNDA + FCGR3BL + GBP-2 | | | 0.86 | 0.96 | 0.73 |

* SS = Sjögren's syndrome; SLE = systemic lupus erythematosus; ROC = receiver operating characteristic; mRNA = messenger RNA. See Table 1 for additional definitions.

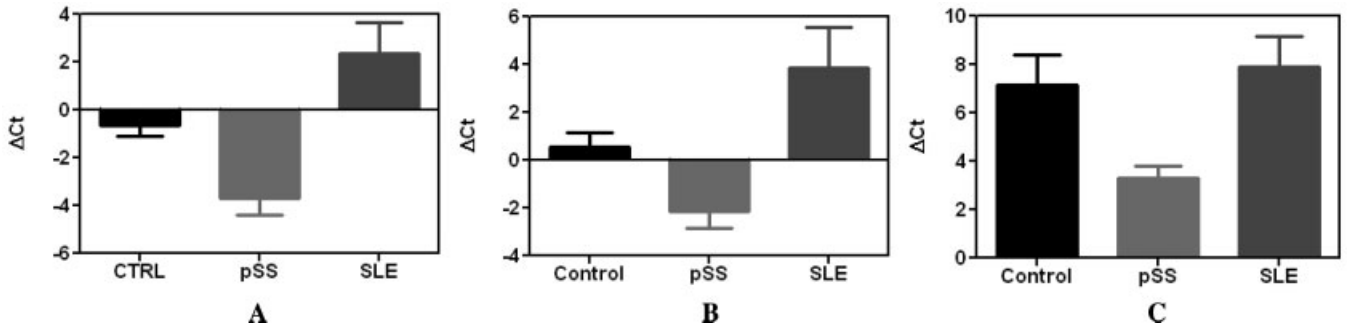


**Figure 2.** Validation of messenger RNA (mRNA) biomarkers **A,** myeloid cell nuclear differentiation antigen, **B,** low-affinity IIIb receptor for the Fc fragment of IgG, and **C,** guanylate binding protein 2, in primary Sjögren's syndrome (pSS; n = 25), systemic lupus erythematosus (SLE; n = 26), and healthy control (CTRL; n = 26) samples. The bar figures indicate the normalized levels of mRNA biomarkers among 3 groups (mean ± SEM). Note that the lower the values for the difference in threshold cycle ($\triangle C_t$), the higher the levels of the mRNA.

Eight candidate mRNA biomarkers were tested in 25 primary SS, 26 SLE, and 26 healthy control samples by real-time quantitative PCR (Table 1). These candidates were previously discovered using microarray profiling and preliminarily verified on the same set of samples (n = 10 for primary SS and n = 10 for control) (14). Three mRNA biomarkers, myeloid cell nuclear differentiation antigen (MNDA), low-affinity IIIb receptor for the Fc fragment of IgG (FCGR3B), and guanylate binding protein 2 (GBP-2), were successfully validated and all were significantly elevated in patients with primary SS (lower $\triangle C_t$ values) compared with healthy controls and patients with SLE (higher $\triangle C_t$ values) (Figure 2), suggesting that they are promising validated biomarkers for primary SS detection (Tables 2 and 3).

## DISCUSSION

In this study, we have successfully validated 3 salivary protein and 3 mRNA biomarkers that can lead to highly sensitive and specific detection for primary SS. These biomarkers not only distinguish patients with primary SS from a healthy control population but, more importantly, can also differentiate patients with primary SS from patients with SLE (as an autoimmune disease control). Regarding the performance to discriminate between patients with primary SS and healthy control subjects, the best biomarkers are $\beta_2$m (ROC 0.95), CPD (ROC 0.88), and MNDA (ROC 0.84). The same 3 biomarkers also exhibit the best performance to discriminate patients with primary SS from patients with SLE (for $\beta_2$m, ROC 0.87; for CPD, ROC 0.82; and for MNDA, ROC 0.79). Combining multiple biomarkers improved the sensitivity and specificity for primary SS detection. For instance, the combination of $\beta_2$m and CPD yielded an ROC value of 0.99, a sensitivity of 100%, and a specificity of 92%, whereas the combination of all 3 protein biomarkers reached an ROC of 0.99, a sensitivity of 94%, and a specificity of 97% (primary SS versus healthy control). In contrast, the combination of all 3 mRNA biomarkers reached an ROC of 0.86, a sensitivity of 88%, and a specificity of 81% (primary SS versus healthy control), which is slightly better than individual mRNA biomarkers. Further addition of mRNA biomarkers to the protein biomarkers does not improve the overall performance (ROC, sensitivity, and specificity) to discriminate patients with primary SS from healthy control subjects. Similarly, the combination of protein biomarkers significantly improved the sensitivity (92%) and specificity (88%) for differentiating patients with primary SS from patients with SLE (ROC 0.94). Although the combination of mRNA biomarkers performed better than individual mRNA biomarkers, the overall performance appeared lower than the combined protein biomarkers (primary SS versus SLE). However, it should be noted that GBP-2 and FCGR3B were highly sensitive biomarkers (92% sensitivity) in distinguishing primary SS from SLE. The best panel of biomarkers for distinguishing patients with primary SS from patients with SLE included $\beta_2$m, MNDA, and GBP-2 and yielded an ROC of 0.95, a sensitivity of 92%, and a specificity of 89%, which was slightly better than the combination of the 3 protein biomarkers (ROC 0.94, sensitivity of 92%, specificity of 88%).

Although CPD and $\alpha$-enolase are novel protein biomarkers, $\beta_2$m was previously detected in patients with primary SS, and the level of salivary (but not serum) $\beta_2$m was highly related to the salivary gland biopsy focus score (19). The value of salivary $\beta_2$m was also evaluated for noninvasive confirmation of the diagnosis of SS (20). Besides protein biomarkers, we have also identified and validated novel mRNA biomarkers. As we discussed previously (14), one of the important findings from our initial microarray profiling was that many up-regulated genes in the saliva of patients with primary SS are involved in the interferon pathway, including the interferon-inducible gene, GBP-2. This gene has a function in cell signaling and was reported to be up-regulated at the mRNA level in minor salivary glands from patients with primary SS (21).

As a result of this study, we have successfully verified a panel of protein and mRNA biomarkers that are highly sensitive and specific for primary SS detection. These biomarkers can discriminate primary SS from SLE, which is an autoimmune disease with a similar immunopathologic background. Nevertheless, these markers may only apply to patients with residual salivary function, and it will be important to test whether they can distinguish patients with primary SS from those with sicca symptoms but no autoimmune disease. Saliva diagnostics offers a combination of low cost, noninvasiveness, and easy sam-

ple collection/processing for disease detection. Testing of these biomarkers in saliva fluids will lead to a simple and noninvasive clinical tool for the diagnosis of primary SS. Verification of CPD and $\alpha$-enolase was based on Western blot analysis in this study. In reality, we would have to develop quantitative assays to measure the absolute levels of these protein biomarkers for diagnostic screening. Of note is that we are developing a point-of-care, saliva-based, microfluidics-based platform for fast and sensitive measurement of these primary SS biomarkers in saliva. This is a worthwhile approach as we recently demonstrated the potential of such a platform for multiplexed measurement of salivary protein and mRNA biomarkers (22). This may lead to a point-of-care diagnostic approach for primary SS in a clinical setting.

## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be submitted for publication. Dr. Hu had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Hu, Gao, Zhou, Kallenberg, Vissink, Wong.

**Acquisition of data.** Hu, Gao, Pollard, Arellano-Garcia, Zhou, Zhang, Kallenberg, Vissink, Wong.

**Analysis and interpretation of data.** Hu, Gao, Pollard, Arellano-Garcia, Zhou, Zhang, Elashoff, Kallenberg, Vissink, Wong.

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