Salivary gland secretome: a novel tool towards molecular stratification of patients with primary Sjögren’s syndrome and non-autoimmune sicca

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ABSTRACT

Objective To explore the potential of salivary gland biopsy supernatants (the secretome) as a novel tool to aid in stratification of patients with sicca syndrome and to study local immunopathology in Sjögren’s syndrome.

Methods Labial salivary gland biopsies were incubated in saline for 1 hour. In these tissue supernatants from a discovery cohort (n=16) of patients with primary Sjögren’s syndrome (pSS) and non-Sjögren’s sicca (nSS), 101 inflammatory mediators were measured by Lumexin. Results were validated in a replication cohort (n=57) encompassing patients with pSS, incomplete SS and nSS.

Results The levels of 23 cytokines were significantly increased in patients with pSS versus nSS in the discovery cohort. These 23 and 3 additional cytokines were measured in a second cohort. Elevated concentrations of 11 cytokines were validated and the majority correlated with clinical parameters. Classification tree analysis indicated that the concentrations of CXCL13, IL-21, sIL-2R and sIL-7Rα could be used to classify 95.8% of patients with pSS correctly.

Conclusion Labial salivary gland secretomes can be used to reliably assess mediators involved in immunopathology of patients with pSS, potentially contributing to patient classification. As such, this method represents a novel tool to identify therapeutic targets and markers for diagnosis, prognosis and treatment response.

INTRODUCTION

Primary Sjögren’s syndrome (pSS) is a chronic, systemic autoimmune disorder characterised by dryness and lymphocytic infiltration of exocrine glands. Salivary gland biopsy is a valuable tool for pSS diagnostics. The lymphocytic focus score (LFS) as enumerated in H&E stained salivary gland tissue slides is part of routine clinical diagnostics and has an important position in current and previous classification criteria. However, this way of analysing tissue has several limitations, including lack of standardisation and poor correlation of the scored abnormalities with dryness.

Optimisation of existing histology protocols to determine salivary gland inflammation will help to improve diagnostics of pSS. In parallel, exploration of other methodologies for analysis of local immunopathology could facilitate diagnostics, prognostics and patient-tailored treatment. Currently used techniques do not allow for high-throughput analyses of protein expression in tissues. Saliva proteomics is a promising tool, but has limitations including inapplicability to the driest patients and technical challenges such as degradation of cytokines by salivary enzymes. We here explored whether multi-cytokine analysis of supernatants from whole minor salivary gland biopsy samples, which we refer to as the ‘secretome’, can be used as a robust and efficient tool for diagnostic, prognostic, and treatment response in patients with sicca.

Key messages

What is already known about this subject?

► Labial salivary gland biopsy plays an important role in Sjögren’s syndrome diagnostics.

What does this study add?

► Salivary gland tissue supernatants allow for detection of a large amount of inflammatory mediators (‘secretome’).

► Robustly increased inflammatory mediators in the secretome of patients with primary Sjögren’s syndrome (pSS) correlate with clinical parameters.

► The salivary gland secretome might be used as an aid to accurately classify patients with pSS.

How might this impact on clinical practice?

► In the future, the salivary gland secretome could be of use to identify therapeutic targets and to develop markers for diagnosis, stratification, prognosis and treatment response in patients with sicca.
as a clinically relevant tool to classify patients and yield insights into immunopathology.

**PATIENTS AND METHODS**

**Patients**

Patients with pSS (n=8 and n=24 in discovery and validation cohort, respectively) were diagnosed by a rheumatologist and fulfilled the American European Consensus Group (AECG) classification criteria. Patients with non-Sjögren’s sicca (nSS) (n=8 and n=17) were defined as patients with sicca complaints, without a connective tissue disease including pSS, without lymphocytic infiltration in the salivary gland biopsy (LFS=0) and without anti-Ro/SSA or anti-La/SSB autoantibodies. Patients with incomplete Sjögren’s syndrome (iSS) (n=16) were defined as patients with sicca complaints, without a connective tissue disease including pSS, who do not fulfill the classification criteria for pSS, but do have signs of lymphocytic infiltration and/or the presence of anti-Ro/SSA or anti-La/SSB autoantibodies (clinical data are described in table 1). Because the tissue supernatants are regarded as rest material and clinical data were provided pseudo-anonymously, no ethical approval or informed consent was required according to the guidelines of the hospital’s ethical committee. Usage of leftover fresh tissue was approved by the hospital’s ethical committee (document nrs. 09–011 and 14–589) and patients gave their written informed consent.

**Salivary gland tissue supernatant preparation and multicytokine analysis**

Fresh labial salivary gland tissues were thoroughly rinsed and incubated with 200 µL of saline (0.9% NaCl) in a 500 µL phial (Sarstedt) for 1 hour at room temperature. The phial with saline was weighed before and after adding the biopsy tissue to determine the weight of the salivary gland biopsy tissue. After 1 hour, the biopsy tissue was transferred to the pathology department for diagnostic procedures. This introduced no changes to routine diagnostic procedures and all tissues could adequately be assessed by the pathologist. The remaining tissue supernatants were rendered cell-free by centrifugation at 500 g for 5 min and stored at −80°C (figure 1A). In tissue supernatants from patients with pSS and nSS/iSS, 101 soluble mediators (online supplementary table 1) were measured by Luminex as previously described. Mediators that were significantly different (p ≤ 0.05) between the two groups were measured in a larger validation cohort by Luminex. Total protein concentrations of the supernatants were measured using a BCA protein assay kit (Thermo Fisher Scientific).

**RNA sequencing**

Of biopsies of 6 patients with nSS, 5 patients with iSS and 12 patients with pSS from this cohort in which the secretome was measured, RNA sequencing was performed, allowing for analyses of correlations between mRNA expression and protein concentrations in the secretome. Retrieval of stored, leftover, frozen tissue samples was approved by the hospital’s ethical committee. Cryopreserved salivary gland tissue was stored in Tissue-Tek at −80°C. Samples were selected based on availability. Sixty sections of 20 µm were cut from each biopsy and lysed in RLT-plus (Qiagen) supplemented with beta-mercaptoethanol. RNA was isolated using the AllPrep Universal Kit (Qiagen). RNA

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**Table 1 Patients’ characteristics**

|                  | Discovery cohort | Validation cohort |
|------------------|------------------|-------------------|
|                  | nSS /iSS (n=6/2) | pSS (n=8)         |
| Gender, n (%)    | 8 (100)          | 7 (88)            |
| Age, years (mean±SD) | 50±17           | 51±13             |
| Autoantibodies   |                  |                   |
| Anti-Ro/SSA+, n (%) | 2 (25)          | 0 (0)             |
| Anti-La/SSB+, n (%) | 0 (0)           | 0 (0)             |
| ANA+, n (%)      | 3 (38)           | 9 (53)            |
| RF+, n (%)       | 0 (0)            | 0 (0)             |
| Lymphocytic focus score (foci/4 mm²) | 0 (0–0)         | 0.2 (0–0.7)      |
| IgA+plasma cells (%) | 73 (70–80)     | 7 (12–25)         |
| Schirmer (mm/5 min) | 2 (0–11)        | 4 (1–7)           |
| Serum IgG (g/L)  | 10.8 (7.6–14.2)  | 11.0 (6.5–12.3)   |
| ESR (mm/hour)    | 7 (5–12)         | 7 (6–10)          |
| ESSDAI score (0–123) | NA            | NA                |
| ESSPRI score (0–10) | NA            | NA                |
| Immunosuppressants, n (%) | 1 (12.5)       | 1 (12.5)          |

*Media with IQR are represented, unless specified otherwise.

ESR, erythrocyte sedimentation rate; ESSDAI, EULAR Sjögren’s Syndrome Disease Activity Index; ESSPRI, EULAR Sjögren’s Syndrome Patient Reported Index; RF, rheumatoid factor; SSA, Sjögren’s syndrome antigen A; SSB, Sjögren’s syndrome antigen B; iSS, incomplete Sjögren’s syndrome; nSS, non-Sjögren’s sicca; pSS, primary Sjögren’s syndrome.*
sequencing was performed on an Illumina NextSeq500 sequencer providing approximately 20 million 75 bp single-ended trimmed reads for each sample. The sample qualities were assessed by FastQC and the reads were mapped to the human genome (GRCh38) using STAR aligner. Since the samples were sequenced in two batches, the batch effect was corrected using RUVSeq package in R. The normalised expression values (vsd normalised read counts) for the genes were calculated using the DESeq2 package in R.

**Statistical analyses**

Statistical analyses were performed in SPSS (V.21). Differences between the groups were assessed by Mann-Whitney U test. Differences between groups were considered statistically significant at p≤0.05. The cytokine concentrations in the validation cohort were used to perform correlations with all available clinical parameters, including LFS, percentage of IgG+/IgM+ plasma cells in the biopsy; Schirmer test results, serum IgG levels, erythrocyte sedimentation rate (ESR), EULAR Sjögren’s Syndrome Disease Activity Index (ESSDAI) and EULAR Sjögren’s Syndrome Patient Reported Index (ESSPRI). Spearman’s rank correlation coefficient was used for correlation analyses. In addition, the cytokine concentrations in the validation cohort were used to perform classification tree analysis using the CHAID (χ² automatic interaction detection) method.

**RESULTS**

**Salivary gland tissue supernatants allow for detection of a large amount of inflammatory mediators**

To assess whether salivary gland supernatants can be used as a tool to study local inflammatory mediators, we measured 101 mediators in 8 patients with pSS and 8 patients with nSS with matched biopsy weights (mean±SD: 51.5±21.2 mg vs 48.7±18.2 mg) by Luminex. Ninety-five of these mediators were detected in all samples and six were detected in the majority of the samples. Twenty-three mediators were significantly increased in the pSS group compared with the nSS group (figure 1B).

**Robustly increased inflammatory mediators in the secretome of patients with pSS correlate with clinical parameters**

We next sought to validate the 23 mediators that were increased in patients with pSS in a validation cohort of 24 patients with pSS, 16 patients with iSS and 17 patients...
with nSS. In addition, IL-21, soluble IL-7Rα and CCL25, which are increased in patients with pSS and play a role in salivary gland inflammation,10–12 were measured in this validation cohort. There were no significant differences between the groups in weights of the biopsies (mean±SD: 63.0±47.6 mg in nSS, 72.7±45.2 mg in iSS and 67.4±28.6 mg in pSS) or in total secreted protein concentrations measured in the supernatants (mean±SD 0.42±0.28 µg/µL in nSS, 0.53±0.24 µg/µL in iSS and 0.55±0.31 µg/µL in pSS) or in the numbers of labial minor salivary glands collected per biopsy (mean±SD: 3.4±2.0 glands in nSS, 4.0±1.6 glands in iSS and 3.9±1.4 glands in pSS).

Eleven mediators were significantly increased in patients with pSS vs patients with nSS: CXCL13, CCL19, CXCL10, TIM-1/KIM-1, IL-31, IL-2, GM-CSF, IL-23, sIL-2R, sIL-7Rα and CCL25 (figures 1B and 2 and (online supplementary figure 1)). In addition, CCL19 was increased in patients with iSS vs patients with nSS (figure 2B). Although trends towards increased cytokine concentrations between patients with pSS and patients with iSS were observed, these did not reach statistical significance. The majority of the differentially expressed cytokines showed correlations with clinical parameters within the patients with pSS including LFS and presence of anti-Ro/SSA auto-antibodies (table 2). CXCL10, CXCL13 and CCL19 significantly correlated with LFS within the pSS group (figure 2B).

Although we anticipated that biopsies with larger total weights might secrete more protein, including cytokines and chemokines, of the cytokines and chemokines that showed significant differences only CCL19 correlated with total tissue weight and total protein (r=0.577, p=0.003 and r=0.497, p=0.013, respectively). Due to the lack of linear correlations between tissue weight or total secreted protein and the levels of most cytokines and chemokines, normalisation to either tissue weight or total secreted protein could not be generally applied. Correction of CCL19 levels for tissue weight (0.21±0.46 pg/mg/mL in pSS vs 0.03±0.04 pg/mg/mL in nSS, p<0.001) or total secreted protein (41.7±72.8 pg/ng/mL in pSS vs 13.1±28.6 pg/ng/mL in nSS, p=0.002) showed a similar significant differences between patients with pSS and patients with nSS. This indicates that the uncorrected data give comparable results to the corrected data. Given this substantiation and the lack of differences in biopsy weights and protein concentrations between the groups, we next confirmed whether the total secreted cytokines reflected actual production as measured by RNA levels of the tissue samples.

To assess whether the increased concentrations of the validated cytokines and chemokines reflect local production, we assessed the association of these cytokines with mRNA expression by RNA sequencing in paired labial salivary gland tissue samples. Indeed, the concentrations of CXCL10, CXCL13 and CCL19 measured in the tissue supernatants correlated with the tissue mRNA expression of these cytokines within the patients with pSS (figure 2B). In addition, IL7R mRNA expression correlated with sIL-7Rα concentrations in all donors (r=0.557, p=0.007). Messenger RNA expression of the other validated cytokines was detectable in <10% of patients, preventing us from assessing reliable correlations with protein levels.

The salivary gland secretome accurately classifies patients with pSS

To investigate whether the secretome can classify patients, decision tree analysis was performed using the expression of all 26 mediators measured in the validation cohort. In an automated and unsupervised manner, a combination of CXCL13, IL-21, sIL-7Rα and sIL-2R was identified to correctly classify 95.8% of patients with pSS and 76.5% of patients with nSS (figure 3). The patient with misclassified pSS had an LFS of 1, no anti-SSA/SSB auto-antibodies and an ESSDAI of 1. There were no differences in clinical or laboratory parameters (Schirmer test, ESR, serum IgG levels) between the patients with correctly and incorrectly classified nSS. Of the patients with iSS, 11 were classified as pSS and five as nSS. The patients with iSS who were classified as pSS had an elevated LFS (0.4±0.3 vs 0.0±0.0, p=0.009) compared with the other patients with iSS.

DISCUSSION

In this study, we show that salivary gland tissue supernatants allow for measurement of a multitude of local proinflammatory mediators. Using this secretome, we demonstrate that patients with pSS have a marked local increase in 11 inflammatory mediators compared with patients with nSS, the majority correlating with clinical parameters. Furthermore, we show that levels of several identified mediators accurately classify patients with pSS and nSS.

All mediators measured were detectable in the majority of patients, indicating that this technique can be used to study a broad range of targets. We observed increased levels of CXCL10, CXCL13 and CCL19 in patients with pSS, all of which are described to be increased in pSS salivary glands according to literature.13–15 Importantly, CXCL13 plays an essential role in germinal centres, and both CXCL13 and the presence of germinal centre-like structures in pSS salivary glands are associated with the risk of lymphoma development.13 16 In addition, we found strong correlations of released cytokine concentrations with gene expression in matched tissue explants, which confirms that the salivary gland tissue is the source of the mediators measured in the secretome. Moreover, the increase of sIL-7Rα and CCL25 in tissue supernatants, which we previously reported, was consistent with salivary gland tissue expression assessed by immunohistochemistry.10 11 17 As such, the secretome is a promising novel tool to study broad panels of proteins and may be useful to assess other mediators including autoantibodies and proteins associated with the activity of tissue cells, such as epithelial cells and fibroblasts.
Figure 2  The secretome reveals multiple significantly upregulated cytokines in the salivary gland of patients with pSS. (A) Twenty-six cytokines were measured in the secretome of the validation cohort consisting of patients with nSS, iSS and pSS using Luminex. Cytokine expression data were quantile normalised around the median. Patients are sorted by subgroup: nSS (LFS 0 anti-SSAneg), iSS (anti-SSA+ and/or LFS>0) and pSS. The patients within the iSS and pSS groups are sorted by LFS (left to right: from low to high). Cytokines are sorted from low to high p value (from top to bottom) in pSS vs nSS, significantly differing cytokines are indicated in bold. (B) The secretome levels of CXCL13, CXCL10 and CCL19 correlate with LFS and with the mRNA expression of their respective gene as measured by RNA sequencing within the patients with pSS (Spearman R, p value). Cytokine concentrations below the detection limit were converted to the lowest point on the calibration curve (lower limit of quantification) multiplied by 0.5 (lower limit of detection). iSS, incomplete Sjögren’s syndrome; LFS, lymphocytic focus score; nSS, non-Sjögren’s sicca; pSS, primary Sjögren’s syndrome; SSA, Sjögren’s syndrome antigen A.
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To properly measure low expressed mediators, the setup of this explant assay could be further optimised. For this study, we used supernatants that were collected without changing standard clinical practice: biopsies for 1 hour in saline at room temperature. With this setup, consisting of multiple salivary glands that were kept for 1 hour in culture medium at 37°C yields significant differences in the production of inflammatory mediators between patients with nSS and pSS (CCL19 and CXCL10, online supplementary figure 2). Hence, we believe that optimisation of the culture protocols will increase the usability of this approach. This should also include assessments of the dynamics of protein expression and production in the secretome samples, also in relationship to protease levels and activity as these could potentially influence detection of certain proteins. Blockade of these proteases might further increase the usability of the secretome assay. In addition, to study which cells release the measured cytokines, and to more precisely quantify their production, culture experiments with isolated cells from biopsies should be performed. As a future perspective, the potential efficacy of therapeutics could be tested by assessment of alterations in the salivary gland secretome.

Using the salivary gland secretome, 95.8% of patients with pSS and 76.5% of patients with nSS could be classified correctly in an unsupervised manner. As such, the secretome could aid in improvement of diagnostics of pSS in the future. The iSS group consists of patients, who are difficult to characterise in clinical practice. They show limited objective signs of Sjögren’s syndrome, but are not diagnosed with pSS by the rheumatologist and do not fulfil the classification criteria. It is relevant to note that 10 out of 16 patients with iSS might have met the 2016 AECG classification criteria if saliva production would have been quantified. Interestingly, the patients with iSS who were molecularly categorised as pSS according to the decision tree analysis had a higher LFS compared with the rest of the group and were anti-Ro/SSA negative. There were no significant differences in cytokine levels between anti-Ro/SSA+ and anti-Ro/SSA- patients with iSS. This indicates that molecular profiling using secretome detects inflammatory activity that was underscored by LFS assessment, which may be of added value in Sjögren’s syndrome diagnosis. However, follow-up of these patients and longitudinal analyses in larger cohorts are needed to show whether the classification on the basis of the salivary gland secretome is clinically relevant for this group of patients.

### Table 2 Correlations of secretome-derived cytokine levels with clinical parameters

|                     | IL-2 | IL-23 | IL-31 | CCL19 (MIP-3β) | CXCL13 (BLC) | CXCL10 (IP-10) | TIM-1/ | GM-CSF |
|---------------------|------|-------|-------|----------------|--------------|----------------|--------|--------|
| Lymphocytic focus score | r    | 0.333 | 0.157 | 0.212          | 0.468        | 0.431          | 0.568  | 0.089  | 0.150  |
|                     | p-value | 0.111 | 0.463 | 0.321          | 0.021        | 0.035          | 0.004  | 0.680  | 0.484  |
| % IgG+/IgM+ plasma cells | r    | 0.325 | 0.044 | 0.107          | 0.101        | 0.140          | 0.299  | 0.124  | 0.276  |
|                     | p-value | 0.151 | 0.850 | 0.643          | 0.663        | 0.546          | 0.188  | 0.592  | 0.226  |
| Schirmer test       | r    | -0.590 | -0.298 | -0.451      | -0.258      | -0.314         | -0.441  | -0.338  | -0.453  |
|                     | p-value | 0.010 | 0.230 | 0.060          | 0.301        | 0.204          | 0.067  | 0.171  | 0.059  |
| ESSDAI              | r    | -0.005 | 0.215 | 0.286          | 0.084        | 0.138          | 0.330  | 0.392  | 0.118  |
|                     | p-value | 0.982 | 0.324 | 0.186          | 0.704        | 0.530          | 0.125  | 0.064  | 0.592  |
| ESSPRI              | r    | 0.118 | -0.156 | -0.186      | 0.022        | -0.392         | 0.184  | -0.11  | 0.244  |
|                     | p-value | 0.700 | 0.610 | 0.544          | 0.942        | 0.185          | 0.548  | 0.971  | 0.423  |
| Serum IgG           | r    | 0.259 | 0.442 | 0.411          | -0.018       | 0.360          | 0.427  | 0.356  | 0.427  |
|                     | p-value | 0.232 | 0.035 | 0.052          | 0.934        | 0.092          | 0.042  | 0.096  | 0.042  |
| ESR                 | r    | 0.150 | 0.154 | 0.439          | 0.410        | 0.331          | 0.131  | 0.400  | 0.081  |
|                     | p-value | 0.493 | 0.483 | 0.036          | 0.052        | 0.122          | 0.552  | 0.058  | 0.715  |
| Anti-Ro/SSA pos vs neg | P value | 0.192 | 0.052 | 0.027          | 0.881        | 0.081          | 0.061  | 0.032  | 0.153  |

Correlations between cytokine concentrations and clinical parameters in the patients with pSS are shown. Spearman r and p values are represented.

Mann-Whitney U test was used to test if cytokine levels significantly differed between anti-SSA+ and anti-SSA- patients with pSS. Significant correlations (p<0.05) are depicted in bold.

Cytokines that did not significantly correlate with any clinical parameter (sIL-2R) or of which correlation have been published (sIL-7R, CCL25) are excluded from this table. No correlations with anti-La/SSB autoantibodies or C4 levels were found.

ESR, erythrocyte sedimentation rate; ESSDAI, EULAR Sjögren’s Syndrome Disease Activity Index; ESSPRI, EULAR Sjögren’s Syndrome Patient Reported Index; SSA, Sjögren’s syndrome antigen A; SSB, Sjögren’s syndrome antigen B; nSS, non-Sjögren’s sicca; pSS, primary Sjögren’s syndrome.

For this group of patients.

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In conclusion, the salivary gland secretome represents a valuable novel tool to measure many local soluble mediators, to provide future insights in immunopathology and potentially aid in diagnostics. This method could be of use to identify therapeutic targets and to develop markers for stratification, prognosis and treatment response in patients with sicca.

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References

1. Fox RL. Sjögren’s syndrome. The Lancet 2005;366:321–31.
2. Vitali C, Bombardieri S, Jonsson R, et al. Classification criteria for Sjögren’s syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002;61:554–8.
3. Shiboski CH, Shiboski SC, Seror R, et al. 2016 American College of Rheumatology/European League Against Rheumatism Classification Criteria for Primary Sjögren’s Syndrome: A Consensus and Data-Driven Methodology Involving Three International Patient Cohorts. Arthritis Rheumatol 2017;69:35–45.
4. Fisher BA, Jonsson R, Daniels T, et al. Standardisation of labial salivary gland histopathology in clinical trials in primary Sjögren’s syndrome. Ann Rheum Dis 2017;76:1161–8.
5. Baldini C, Cecchettini A, Gallo A, et al. Updates on Sjögren’s syndrome: from proteomics to protein biomarkers.
6. de Jager W, te Velthuis H, Prakken BJ, et al. Simultaneous detection of 15 human cytokines in a single sample of peripheral blood mononuclear cells. Clin Diagn Lab Immunol 2003;10:133–9.
7. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15–21.
8. Risso D, Ngai J, Speed TP, et al. Normalization of RNA-seq data using factor analysis of control genes or samples. Nat Biotechnol 2014;32:896–902.
9. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.
10. Hillen MR, Blokland SL, Risselada AP, et al. High soluble IL-7 receptor expression in Sjögren’s syndrome identifies patients with increased immunopathology and dryness. Ann Rheum Dis 2016;75:1735–6.
11. Blokland SLM, Hillen MR, Kruize AA, et al. Increased CCL25 and T Helper Cells Expressing CCR9 in the Salivary Glands of Patients With Primary Sjögren’s Syndrome: Potential New Axis in Lymphoid Neogenesis. Arthritis Rheumatol 2017;69:2038–51.
12. Kwok SK, Lee J, Yu D, et al. A pathogenic role for IL-21 in primary Sjögren’s syndrome. Nat Rev Rheumatol 2015;11:368–74.
13. Barone F, Bombardieri M, Rosado MM, et al. CXCL13, CCL21, and CXCL12 expression in salivary glands of patients with Sjögren’s syndrome and MALT lymphoma: association with reactive and malignant areas of lymphoid organization. J Immunol 2008;180:5130–40.
14. Ogawa N, Ping L, Zhenjun L, et al. Involvement of the interferon-gamma-induced T cell-attracting chemokines, interferon-gamma-inducible 10-kd protein (CXCL10) and monokine induced by interferon-gamma (CXCL9), in the salivary gland lesions of patients with Sjögren’s syndrome. Arthritis Rheum 2002;46:2730–41.
15. Carubbi F, Alunno A, Cipriani P, et al. Is minor salivary gland biopsy more than a diagnostic tool in primary Sjögren’s syndrome? Association between clinical, histopathological, and molecular features: a retrospective study. Semin Arthritis Rheum 2014;44:314–24.
16. Risselada AP, Locche MF, Kruize AA, et al. The role of ectopic germinal centers in the immunopathology of primary Sjögren’s syndrome: a systematic review. Semin Arthritis Rheum 2013;42:368–76.
17. Bikker A, Kruize AA, Wenting M, et al. Increased interleukin (IL)-7Rα expression in salivary glands of patients with primary Sjögren’s syndrome is restricted to T cells and correlates with IL-7 expression, lymphocyte numbers and activity. Ann Rheum Dis 2012;71:1027–33.
18. Hartkamp LM, Fine JS, van Es IE, et al. Btk inhibition suppresses agonist-induced human macrophage activation and inflammatory gene expression in RA synovial tissue explants. Ann Rheum Dis 2015;74:1603–11.
19. Lubberts E, Joosten LA, Chabaud M, et al. IL-4 gene therapy for collagen arthritis suppresses synovial IL-17 and osteoprotegerin ligand and prevents bone erosion. J Clin Invest 2000;105:1687–710.

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FIGURE 3 Classification tree analysis reveals that combinations of secretome-derived cytokines can be used to classify patients. Classification tree analysis was performed using the CHAID method. Concentrations of CXCL13, IL-21, sIL-7Rα and sIL-2R were identified to distinguish patients with nSS from pSS correctly overall in 87.8% of cases. nSS, non-Sjögren’s sicca; pSS, primary Sjögren’s syndrome.

Table 3 Observed and predicted classification for the CHAID applied with CXCL13 and IL-21 as the root node.

| Classifications | Predicted | Correct |
|-----------------|-----------|---------|
| nSS             | 13        | 23      |
| pSS             | 4         | 9       |
| Overall Accuracy| 87.8%     |         |