Identification of parotid salivary biomarkers in Sjögren’s syndrome by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional difference gel electrophoresis

O. H. Ryu¹, J. C. Atkinson², G. T. Hoehn⁴, G. G. Illei³ and T. C. Hart¹,²

Objectives. To identify the most significant salivary biomarkers in Sjögren’s syndrome (SS) using proteomic methods.

Methods. Parotid saliva from 20 non-SS subjects and 41 primary SS patients was analysed. Protein expression profiles for each sample were generated by surface-enhanced laser desorption/ionization time-of-flight-mass spectrometry (SELDI-TOF-MS). Mean peak intensities of SS patients and non-SS subjects were compared by univariate analyses. Samples pooled by diagnosis (SS and non-SS) and labelled with different Cy dyes were compared by two-dimensional difference gel electrophoresis (2D-DIGE). Two protein levels that were most significantly different by SELDI-TOF-MS and 2D-DIGE were validated by enzyme-linked immunosorbent assay in individual samples.

Results. SELDI-TOF-MS of 10–200 kDa peaks revealed eight peaks with >2-fold changes in the SS group that differed from non-SS at P < 0.005. Peaks of 11.8, 12.0, 14.3, 80.6 and 83.7 kDa were increased, while 17.3, 25.4, and 35.4 kDa peaks were decreased in SS samples. 2D-DIGE identified significant increases of β-2-microglobulin, lactoferrin, immunoglobulin (Ig) κ light chain, polymeric Ig receptor, lysozyme C and cystatin C in all stages of SS. Two presumed proline-rich proteins, amylase and carbonic anhydrase VI, were reduced in the patient group. Three of these ten biomarkers have not been associated previously with SS.

Conclusions. The salivary proteomic profile of SS is a mixture of increased inflammatory proteins and decreased acinar proteins when compared with non-SS. Future studies will test the ability of these biomarker levels, alone and in combination, to diagnose the salivary component of SS.

Key words: Sjögren’s syndrome, Saliva, Proteomics, 2D-DIGE, SELDI-TOF-MS.

Sjögren’s syndrome (SS), an autoimmune disease characterized by lymphoplasmocytic infiltration of the salivary and lacrimal glands, occurs in the absence (primary SS) or presence (secondary SS) of another major connective tissue disease. One-third of patients with primary SS develop systemic extraludinal manifestations, including malignant lymphoma [1, 2]. Serious outcomes occur more frequently in patients with decreased serum complement fraction 3 and 4, palpable purpura and the presence of mixed cryoglobulins [1–3], reinforcing the need for early diagnosis of SS.

The modified European classification criteria include a minor salivary gland biopsy [4]. Lymphoplasmocytic infiltration can be semiquantified with focus scores, and a focus score ≥1 is required to diagnosis primary SS in patients without anti-SS-A or anti-SS-B [4]. Unstimulated whole salivary flow rates have a low specificity for SS [5]. However, there are limitations with biopsies. The procedure can cause permanent dysesthesia of the lip, and it isdifficult to ask patients to have repeat biopsies to assess disease progression or therapeutic responses. Focus scores also can be negative in patients fulfilling the diagnostic criteria for SS [4].

The simultaneous measurement of large numbers of expressed proteins, known as proteomic profiling, is becoming an important screening tool for identifying disease biomarkers [6–9]. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) is a highly sensitive method that detects minute protein differences between individual biological samples. In SELDI-TOF-MS, crude samples are loaded on ProteinChips having various surface characteristics such as ionic, hydrophobic and metal-affinity. Bound proteins are detected by TOF mass spectrometry, and peak intensities of the mass spectra that correspond to relative protein abundance of different patient groups are compared. SELDI-TOF-MS permits high-throughput analysis of multiple clinical samples, such as serum, urine and other biological fluids [10–12].

Disease-associated biomarkers detected by SELDI-TOF-MS must be identified using other methods. Two-dimensional difference gel electrophoresis (2D-DIGE) allows comparison of changes in protein abundance across multiple samples simultaneously with minimal gel-to-gel variation. Samples labelled with different fluorescence dyes are separated in one gel, and protein expression is quantified and compared using fluorescence intensity within a single gel or across multiple gels. Compared with conventional 2D gels, 2D-DIGE can generate reproducible data and has the
potential for high-throughput analysis. Changes in abundance are detectable across a linear range of four orders of magnitude [13, 14].

Many changes in SS salivary constituents have been described previously, suggesting that saliva could be used to diagnose the syndrome. Increased concentrations of Na+, Cl [5, 15, 16], IgG [16], lysozyme [17], matrix metalloproteinase (MMP)-2 and MMP-9 [18] in parotid saliva; lactoferrin [19–21], IgA [16, 22, 23], β2-microglobulin [24–27], albumin [16, 23] in both parotid and whole saliva; and kallikrein [28] and cystatins C and S [23] in whole saliva have been reported. The present study compared the salivary proteomes of SS and non-SS patient groups using modern proteomic methods (SELDI-TOF-MS and 2D-DIGE) to identify the most significantly different salivary biomarkers for future studies testing their diagnostic potential.

Material and methods

Reagents

Cy dyes, immobilizes IPG strips, α-cyano-4-hydroxycinnamic acid and chemicals for 2D gel electrophoresis were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). The trypsin and calibrant mixture for the 4700 Proteomics Analyzer were from Promega (Madison, WI, USA) and Applied Biosystems (Foster City, CA, USA), respectively. ProteinChips and sinapinic acid were purchased from Ciphergen (Fremont, CA, USA) and Sigma, respectively. The enzyme-linked immunosorbent assay (ELISA) kit for lactoferrin was from Calbiochem (La Jolla, CA, USA) and that for β2-microglobulin from R&D systems (Minneapolis, MN, USA).

Patient selection and saliva collection

Parotid saliva samples were collected from all 41 primary SS patients studied, and from 20 sex- and age-matched controls, including 15 non-SS subjects with complaints of xerostomia who did not meet diagnostic criteria for SS and five healthy volunteers. The SS patients were further divided into two groups by focus score [29]. Twenty SS patients were placed in the low/medium focus score group (focus score of 1–5, mean focus score ± S.D. = 3.4 ± 1.4) and 21 SS patients into the medium/high focus score group (focus score of 6–12, mean focus score ± S.D. = 9.0 ± 2.6). The mean focus score ± S.D. for the non-SS group was 0.9 ± 0.8. Written informed consent was obtained from all subjects, and the study was approved by the Institutional Review Board of the National Institute of Dental and Craniofacial Research. Every subject had an evaluation of disease duration.

Saliva samples were collected between 9 and 11 a.m. from patients who had refrained from eating or drinking for 2 h. Unstimulated salivary flow rates were determined. After that, the tongue was swabbed bilaterally every 30 s with 2% citric acid to stimulate flow. After 2 min, parotid saliva was collected on ice for 1 min using a Carlson-Crittenden collector and stored at −80°C. Unstimulated whole saliva flow rates (mean ± s.d.) of the groups were 0.045 ± 0.095 (all SS), 0.028 ± 0.060 ml/min (low/medium focus), 0.067 ± 0.157 ml/min (medium/high focus) and 0.263 ± 0.287 ml/min (non-SS). Total group mean stimulated parotid flows (ml/min/gland) were 0.245 ± 0.184 (all SS), 0.292 ± 0.197 (low/medium focus score), 0.201 ± 0.180 (medium/high focus score) and 0.472 ± 0.256 (non-SS).

Sample preparation, protein profiling and data analysis for SELDI-TOF-MS

Thawed samples were centrifuged at 13000 r.p.m. for 5 min to remove insoluble material, and all procedures were performed at 4°C. Q10 anion exchange ProteinChip (Ciphergen Biosystems Inc., Fremont, CA, USA) surfaces were equilibrated with 150 μl of binding buffer (100 mM Tris- HCl, pH 9.0). In preliminary evaluations of four different chips, the Q10 anion chip bound parotid proteins with the greatest reproducibility. Individual saliva samples were mixed with denaturing buffer (9 ml urea and 2% CHAPS) at a ratio of 2:3. Each of the denatured samples (10 μl of each) was applied in duplicate with 90 μl of binding buffer to the pre-equilibrated Q10 ProteinChips. ProteinChip arrays were incubated for 60 min at room temperature with vigorous shaking, washed twice with binding buffer for 5 min each, followed by two washes with distilled water. Arrays were dried at room temperature for 15 min, followed by two additions (1 μl each) of a 50% solution of sinapinic acid, prepared in 50% acetoneitrile and 0.5% trifluoroacetic acid (TFA). Sample handling, including deposition of matrix, was performed on a Biomek 2000 automated work station (Beckman-Coulter, Thousand Oaks, CA, USA) using two 96-well BioProcessors (Ciphergen). To quantify chip to chip variation within an experiment, the same pooled saliva sample containing both non-SS and patient saliva was included on each ProteinChip array. Samples were analysed using SELDI-TOF-MS (Protein Biology System II, Ciphergen Biosystems). All spectra consisted of 130 averaged laser shots and were externally calibrated using All-in-One Protein Standard II (Ciphergen Biosystems), containing seven calibrants between 7 and 147 kDa. Spectral data were processed similarly using CiphergenExpress 3.1 data management software. Spectra were mass aligned, baseline-subtracted using a smoothing feature and a fitting width of eight times expected peak width, and then normalized by total ion current. Peaks with signal to noise ratios of greater than three and valley depths greater than three were automatically detected using the Biomarker Wizard peak picking algorithm. Univariate analyses (Mann–Whitney non-parametric) t-tests were used to compare the mean-intensity values of each recognized peak at the molecular mass range of 10–200 kDa, and P values of each cluster comparison were generated.

Sample preparation for 2D gel electrophoresis

Aliquots from the individual salivary samples were pooled into various groups for analyses by 2D gel electrophoresis. The first set of 2D gels compared a group of pooled non-SS samples (20 samples) with a group containing all SS samples (41 samples). A second set of gels was performed using three groups of pooled saliva: non-SS (20 samples), SS patients with low/medium focus scores (20 samples) or SS patients with medium/high focus scores (21 samples). Salivary proteins were precipitated with absolute ethanol and resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS and 15 mM Tris, pH 8.5) with 1/10 of original salivary volume. Protein concentration was determined by the Bradford method (Bio-Rad, Carlsbad, CA, USA) using bovine serum albumin as a standard protein.

Two-dimensional differential gel electrophoresis (2D-DIGE): dye-labeling, imaging and data analysis

Equal amounts of pooled protein samples were minimally labelled with Cy2, Cy3 and Cy5 dye. Reactions were quenched by adding 10 mM lysine. All reactions were performed in the dark and on ice. For isoelectricfocusing (IEF), Cy-dye-labelled samples were mixed together and reconstituted with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% dithiothreitol, 2% pH 3–10
pharmalyte and trace amounts of bromophenol blue). The mixed sample was loaded on an immobilized pH 3–10 non-linear gradient IPG strip of 7 cm, and then run using an Ettan IEF system. After focusing, strips were equilibrated for 15 min in a solution containing 6M urea, 30% glycerol, 2% sodium dodecyl sulphate (SDS), 100 mM Tris (pH 8.0), trace amounts of bromophenol blue and 10 mg/ml dithiothreitol (DTT) followed by a second 15-min equilibration with iodoacetamide (25 mg/ml) instead of DTT. Strips were rinsed in a 1 x 2-(N-morpholino)ethanesulfonic acid (MES) SDS-polyacrylamide gel electrophoresis (PAGE) buffer, applied to a 12% NuPAGE gel and electrophoresed at 120 V. Cy dye images were collected using a 9400 Typhoon scanner (Amersham) in a fluorescence mode at a pixel size of 100 μm. Cy2, Cy3 and Cy5 images were scanned using 488, 532 and 633 nm lasers, respectively, and an emission filter of 520, 580 and 670 nm bandpass filters, respectively. DeCyder V 5.0 (Amersham) was used for quantitative spot analysis. Gel image pairs were processed by the DeCyder batch processor (BP) and biological variation analysis (BVA) modules to quantify differences in volume ratios using t-test analyses. The DeCyder differential in-gel (DIA) module was used for pairwise comparisons of protein abundance in non-SS, low/medium focus SS patient and medium/high focus SS patient samples. Changes in protein abundance were calculated as a fold increase or decrease in volume ratio. Fold changes were calculated as a mean and standard deviation with four gel pairwise DIA comparisons. Statistical significance was determined by the Student’s t-test.

**Gel staining**

Proteins were visualized by Coomassie Blue staining using a SimpleBlue SafeStain kit (Invitrogen, Carlsbad, CA, USA). Briefly, the gel was stained with 0.1% Coomassie Brilliant Blue (CBB) G-250 with a modified Neuhof solution [30] to identify proteins for removal and subsequent matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). To detect proline-rich proteins, gels were stained with 0.1% CBB R-250 in a 40% ethanol and 10% acetic acid solution for 5h, followed by several days’ incubation in 10% v/v acetic acid destain to visualize pink–violet bands [31].

**Protein identification with MALDI-TOF-MS**

Spots of interest were picked from gels and processed by a fully automated Spot Handling Workstation (Amersham). Gel plugs were washed with 50 mM ammonium bicarbonate–50% methanol, followed by 50% acetonitrile–0.1% TFA and finally 90% acetonitrile for drying. After trypsin digestion in 20 mM ammonium bicarbonate, extracted peptides were dried and resuspended in 2% acetic acid destain to visualize pink–violet bands [31].

Protein identification was further validated manually through BLAST using the SwissProt database (National Center for Biotechnology Information).

**ELISA**

Lactoferrin and β2-microglobulin concentrations were determined from aliquots of individual patient samples used in the experiments above by ELISA using the manufacturers’ protocols.

**Results**

**Selection of differentially expressed proteins by SELDI-TOF-MS**

Three samples (one patient from the SS low/medium focus group, one patient from the medium/high group and one non-SS) were excluded from the analyses because of inconsistent sample binding. We only considered peaks with >10000 Da to match the molecular weight (MW) range of the 2D-DIGE gels used in these experiments. A total of 81 peaks were detected. Peak values were generated for each sample. Mean peak intensities of the groups were compared with univariate analyses. Thirteen peaks in the SS patient group were significantly different from non-SS (P <0.01). Eight were selected as biomarker candidates as there was a >2-fold difference in mean intensities of the SS peaks when compared with non-SS, and all differed at P <0.005 (Table 1). Five peaks were increased in the SS group (11.8 kDa, +3.0-fold, P = 1.6 x 10^-6; 12.0 kDa, +2.2-fold, P = 3.1 x 10^-6; 14.3 kDa, +2.0-fold, P = 1.3 x 10^-3; 80.6 kDa, +5.1-fold, P = 1.0 x 10^-5; and 83.7 kDa, +2.7-fold, P = 1.2 x 10^-3), and three peaks (25.3 kDa, -2.0-fold, P = 3.6 x 10^-4; 17.3, -2.1-fold, P = 3.8 x 10^-3; and 35.4 kDa, -3.8-fold, P = 3.8 x 10^-3) were decreased. The four peaks that differed most significantly are shown in Fig. 1A. A gel view pattern for two peaks is shown in Fig. 1B.

**Determination of differentially expressed proteins by 2D-DIGE**

Final concentrations of pooled precipitated proteins were 3.1 mg/ml (non-SS) and 3.4 mg/ml (SS, Fig. 2A). Protein abundance of the non-SS group in each gel was normalized to compare with that of the SS group. Seven proteins in SS parotid saliva had a volume ratio change of >1.5-fold (either increased or decreased) that differed from non-SS at P < 0.05 (Table 2, Fig. 2B, labelled as 1–7). Three additional proteins detected by 2D-DIGE with a volume ratio change of at least >1.3-fold in the SS group were included as biomarkers for future study (labelled as 8–10 in Fig. 2B). The significance levels for all three proteins was less than 0.1 (protein 8, P = 0.062; protein 9, P = 0.092; protein 10, P = 0.059). We used a lower threshold of change to select protein candidates from 2D-DIGE gels than was used in SELDI-TOF-MS as 2D-DIGE ratio changes were uniformly smaller for all detected proteins. A graphic display of the protein with the greatest increase (protein 1) and decrease (protein 3) in volume change is given in Fig. 2C.

One 2D-DIGE gel was stained with CBB G-250 to visualize protein spots for removal and mass analysis; this only stained a portion of the proteins labelled by fluorescent dyes. Proteins 1, 2, 5, 6 and 7, 8, 9 and 10 (Fig. 2B, a) were removed and identified as β2-microglobulin (1), lactoferrin (2), Ig κ light chain (5), polymeric Ig receptor (pIgR, 6), salivary amylase (7), lysozyme C (8), carbonic anhydrase VI (9) and cystatin C (10). Of these proteins, six were increased in the SS groups (β2-microglobulin, lactoferrin, Ig κ light chain, pIgR, lysozyme C, carbonic anhydrase VI), and one (Ig κ light chain) was decreased. The significance levels for all three proteins was less than 0.1 (protein 8, P = 0.062; protein 9, P = 0.092; protein 10, P = 0.059).
FIG. 1. Protein profiles of salivary samples by SELDI-TOF-MS using Q10 ProteinChips in the molecular range of 10–200 kDa. (A) An intensity plot of protein peaks with \( P \) value <0.0006 and >2-fold changes in non-SS and SS patient groups. Data are expressed as intensity, in arbitrary units quantifying each protein peak. (B) Software-generated gel-view format of two proteins with highly significant changes in the SS patients (11.8 and 80.6 kDa peaks).

TABLE 1. Biomarkers identified as different in Sjögren’s syndromes parotid saliva by SELDI-TOF-MS and/or 2D-DIGE

<table>
<thead>
<tr>
<th>SELDI-TOF-MS</th>
<th>2D-DIGE</th>
<th>Protein identification from database sequence match</th>
<th>Protein candidatesa [reference]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass ratio</td>
<td>Change</td>
<td>Mass ratio</td>
<td>Change</td>
</tr>
<tr>
<td>(kDa)</td>
<td>(fold)</td>
<td>(kDa)</td>
<td>(fold)</td>
</tr>
<tr>
<td>80.6</td>
<td>+5.1</td>
<td>80.2</td>
<td>+2.2</td>
</tr>
<tr>
<td>11.8</td>
<td>+3.0</td>
<td>11.6</td>
<td>+2.3</td>
</tr>
<tr>
<td>83.7</td>
<td>+2.7</td>
<td>84.4</td>
<td>+1.5</td>
</tr>
<tr>
<td>35.4</td>
<td>–2.7</td>
<td>35.4</td>
<td>–1.4</td>
</tr>
<tr>
<td>25.3</td>
<td>–2.5</td>
<td>25.3</td>
<td>–2.5</td>
</tr>
<tr>
<td>12.0</td>
<td>+2.2</td>
<td>12.0</td>
<td>+2.2</td>
</tr>
<tr>
<td>17.3</td>
<td>–2.1</td>
<td>17.5</td>
<td>–2.5</td>
</tr>
<tr>
<td>14.3</td>
<td>+2.0</td>
<td>14.6</td>
<td>+1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.0</td>
<td>–1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.7</td>
<td>+1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56.4</td>
<td>–1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.3</td>
<td>+1.3</td>
</tr>
</tbody>
</table>

Protein candidates were selected based on reported salivary proteins within ±3% of SELDI-TOF-MS molecular mass peaks. PRB; salivary proline-rich glycoprotein precursor.

\( ^a \)Mass unit is kDa.
FIG. 2. Analysis of differentially expressed protein profiles in parotid saliva from Sjögren’s syndrome (SS) patient and non-SS groups. (A) Schematic diagram illustrating the 2D-DIGE method and data analysis. Step 1: Sample preparation and protein quantification. Step 2: Four different DIGE gels were designed by labelling pooled salivary samples from non-SS and SS patients with three different Cy dyes. Proteins were separated by 2D gel and Cy dye images were collected using a fluorescence scanner. Step 3: Student’s t-test of protein signals between non-SS and SS patients were performed by DeCyder batch processor (BP) and BVA modules. Image analyses by image view and 3D view display the gel images and three dimensions of a selected spot for non-SS and diseased samples. The graph view represents a graph of protein abundance for a single spot across the four different images in the analysis set. Dotted lines with circular points indicate data from each gel and the solid line with plus signs shows the average value from four gels. The table view module provides the average ratio (i.e. mean of the average abundance of protein in the disease group/average abundance of protein in the non-SS group) of a selected spot as well as the statistical significance of the difference. Step 4: Differentially expressed proteins are identified by comparison of MALDI-TOF-TOF peptide mass fingerprinting data with human protein database. (B) Detection of protein spots by various staining methods. (a) DIGE: 10 differentially expressed proteins as detected by fluorescence are circled; (b) after CBB R-250 and acetic acid destaining the two spots not stained by CBB G-250 turned pink suggesting they were proline-rich proteins. (C) Graph view of the two protein spots with the most significant changes detected by DIGE. Protein abundance of the SS patients was compared with non-SS patients. Dotted lines indicate which spots are compared in the standard display method of DeCyder graphing software. The control spot (in this case, non-SS) is arbitrarily set at 0 (log 1). The plot demonstrates how the SS patients differed from control for that particular protein in each of the gels. The mean value for the SS group is represented by a cross.
and cystatin C), while two were decreased (salivary amylase and carbonic anhydrase VI).

Proteins 3 and 4 were not visible with CBB G-250 staining. We suspected that these proteins were proline-rich proteins (PRPs) based on their MW, their known abundance in parotid saliva and their lack of staining with the CBB G-250 method. Therefore, another 2D-DIGE gel was stained with CBB R-250 combined with a 10% acetic acid destain to visualize PRP in gels [31]. This technique stained protein spots 3 and 4 pink, suggesting they were PRPs (Fig. 2B, b). Their mass data did not match any protein in the database.

**Comparison of expression levels in non-SS and SS patients with low/medium focus score or medium/high focus score**

Protein profiles of the SS low/medium focus group and medium/high focus group were compared with each other and with non-SS. Equal sample volumes (25 μl) were labelled with different Cy dyes and separated on four individual gels using 2D-DIGE (Fig. 3A).

Although similar protein changes were found in both patient groups when compared with non-SS, there was little difference in expression levels of proteins of the two patient groups (Fig. 3B). Statistically significant increases in β2-microglobulin (+2.9-fold low/medium focus; +2.4-fold medium/high) and lactoferrin (+3.8-fold low/medium focus; +3.6-fold medium/high) were found in both groups when compared with non-SS. Decreases of presumed proline-rich proteins were slightly greater in SS patients with medium/high focus scores (−3.2-fold for protein 3 and −2.9-fold for protein 4) than those with low/medium scores (−2.8-fold for protein 3 and −2.4-fold for protein 4) when compared with control. No other differences were detected by 2D-DIGE in levels of the other six biomarkers (Table 2).

**β2-Microglobulin and lactoferrin by ELISA**

Levels of β2-microglobulin and lactoferrin were further validated by ELISA using aliquots from the individual samples. Levels of β2-microglobulin were +4.3-fold for the low/medium focus group and +3.7-fold for the medium/high group. β2-Microglobulin levels exceeded the mean + 2 S.D. of non-SS values in 50.0 and 31.6% of low/medium focus and medium/high focus patients, respectively (Fig. 4A). Lactoferrin concentrations were +3.7- and +3.6-fold in low/medium focus and medium/high focus patients, respectively, and 80.0 and 78.9% of both groups exceeded the mean + 2 S.D. of non-SS values (Fig. 4C). Other salivary biomarkers were not validated by ELISA because of insufficient amounts of sample.

**Discussion**

SELDI-TOF-MS is a high-throughput method that compares expression levels of hundreds of individual proteins from multiple samples in parallel [8–10]. Its strengths are its ease of sample preparation and high-throughput capabilities, but it does not identify proteins or allow absolute protein quantification. Two-dimensional gel electrophoresis can compare expression levels of hundreds of individual proteins from multiple samples in parallel [8–10]. Its strengths are its ease of sample preparation and high-throughput capabilities, but it does not identify proteins or allow absolute protein quantification.

Two-dimensional gel electrophoresis can compare expression levels of hundreds of individual proteins from multiple samples in parallel [8–10]. Its strengths are its ease of sample preparation and high-throughput capabilities, but it does not identify proteins or allow absolute protein quantification.
each sample is analysed in a separate gel. High gel-to-gel variation makes detection of corresponding spots unreliable, and the quantification of differences is difficult because of the high variability of traditional staining methods.

We used these two protein quantification methods to compare the parotid salivary proteome of non-SS and SS subjects. The purpose was to identify proteins that differed most significantly between the groups as an initial step for the development of saliva-based diagnostic tests for use in patients with complaints of dryness. Therefore, protein profiles of individual patients and groups with all levels of salivary disease activity were compared with a group that primarily contained patients with dryness complaints that did not meet diagnostic criteria. Using this approach, 10 biomarkers were identified in the SS group, three of which had not been described previously.

In our study, 2D-DIGE gel analysis revealed more than 100 parotid protein spots that differed in molecular mass and pI (isoelectric point) values. Analyses demonstrated that lactoferrin and β2-microglobulin showed the greatest increases in SS patients. These findings were validated by ELISA. Lactoferrin is a product of intercalated ductal cells and scattered acinar cells in the parotid gland [32]. Previous studies of SS saliva report increases in lactoferrin [19–21] without a clear association with the amount of lymphocytic infiltration. Since lactoferrin is increased in other

![Figure 3](https://academic.oup.com/rheumatology/article-abstract/45/9/1077/2255597/1083)
diseases affecting salivary glands, including parotitis [19] and diabetes [33], it cannot be used alone to diagnose the salivary component of SS. β2-Microglobulin, the light-chain molecule of the major histocompatibility complex class I antigen [34], is present on the membrane surface of many nucleated cells, including infiltrating lymphocytes and salivary gland epithelium [35]. Increased levels of this protein in SS saliva may relate to salivary gland inflammatory activity, rather than lymphocyte number, as no association was found between β2-microglobulin concentrations and focus scores.

Increases of polymeric Ig receptor (pIgR, also known as secretory component) and Ig κ light chain were also detected in SS patients. Poly Ig receptor (pIgR) transports polymeric immunoglobulins through salivary epithelia into saliva. Its expression is regulated by microbial products through Toll-like receptor signalling, and by hormones and cytokines [36, 37] such as interferon (IFN)-gamma and tumour necrosis factor (TNF)-alpha, which are increased in SS salivary glands [38]. The increased Ig κ light chains in SS saliva with an absence of elevated albumin (which accompanies serum leakage) probably reflects the increased intra-glandular immunoglobulin synthesis of the disease [16, 22, 23]. We also detected increases of lysozyme C (about 1.4-fold) and cystatin C (about 1.3-fold), which have been reported previously [17, 23].

FIG. 4. Quantification of β2-microglobulin and lactoferrin by ELISA. (A) Comparison of amounts of β2-microglobulin in non-SS, low/medium focus score (SS low/med) and medium/high focus score Sjögren’s syndrome (SS med/high) patient groups. (B) Plot of β2-microglobulin concentrations by focus score (● = non-SS and ○ = SS). (C) Comparison of amounts of lactoferrin in non-SS, low/medium focus and medium/high focus SS patient groups (● = non-SS and ○ = SS). (D) Plot of lactoferrin concentrations by focus score.

Four proteins, two purported proline-rich proteins, salivary amylase and carbonic anhydrase, were decreased in the SS salivary profile. Proline-rich proteins, major constituents of parotid saliva, have a predominance of the amino acids proline, glycine and glutamic acid [31, 39–42]. In 2D-DIGE, fluorescence labelling occurs through lysine residues, allowing easy detection of PRPs with this method. We suspected that proteins 3 and 4, detected by fluorescence in the 2D-DIGE gels, were PRPs after discovering that these protein spots stained pink using a method optimized for visualization of PRPs [31]. The locations of the pink spots were similar to those reported for PRPs on 2D gels [41, 42]. Two other unmatched SELDI-TOF-MS peaks may be other PRPs (Table 1). Decreases in PRP may reflect acinar damage in SS glands, as the volume ratios of presumed PRPs were greatest in the SS patients with higher focus scores. Consistent with a previous report [16], the decrease in amylase in the SS group suggests acinar parenchymal damage. Finally, the decrease in carbonic anhydrase (CA) VI found in our study agrees with a recent report of its decreased gene expression in SS minor gland biopsies [43]. CA VI, serous acinar cell product, is the only secretory isoform in the CA gene family [44]. It is part of the salivary buffering system, which protects teeth from demineralization and caries.

We found no association between focus scores and any biomarker, and the scores were evenly distributed from 1 to 12.
Parotid salivary biomarkers in Sjögren’s syndrome by SELDI-TOF-MS and 2D-DIGE

(Figs 4B and 4D). Focus scores assess lymphocyte number, not activity, and do not correlate strongly with salivary flow rates [45]. In fact, the mean unstimulated flow rate of SS patients with fewer foci was less than in the high focus score group, though their stimulated flow rate was higher. Salivary biomarkers in glandular saliva may prove better markers of salivary gland disease activity in SS than focus score. Future studies should relate absolute values of salivary biomarkers (such as ng/ml as determined by ELISA) to flow rates. These calculations are not possible with SELDI-TOF-MS and 2D-DIGE results as data are expressed in arbitrary units. Multiplying these values by flow rate to calculate output/minute is invalid.

Our findings indicate the SS salivary protein profile is a mixture of increased inflammatory proteins and decreased acinar proteins, consistent with the recently published tear proteomic pattern of SS [46]. In that study, 10 biomarkers were detected using SELDI-TOF-MS, which does not provide protein identity. Seven were decreased and three were increased in SS, demonstrating that secretory protein loss is an important characteristic of this disease. Therefore, future studies of SS saliva and salivary glands should also identify decreased proteins, as these losses could relate to the significant oral diseases of this patient group. The biomarkers identified in this study need to be validated in an independent, larger set of SS patients, non-SS and healthy controls to establish their clinical utility for the diagnosis, monitoring and management of SS. Other studies should apply this technology to examine changes in salivary proteins in relation to secretory function in SS, and include saliva from the submandibular/sublingual glands that are severely affected in SS [5].

Acknowledgements

We acknowledge support from the Intramural Program of the NIDCR/NIH, guidance from Bruce Baum, and technical support from Rong-Fong Shen, Wells W. Wu and Angelis Aponte from the Proteomics Core facility of the NHLBI/NIH.

The authors have declared no conflicts of interest.

Reference


