Original

# Discovery of putative salivary biomarkers for Sjögren's syndrome using high resolution mass spectrometry and bioinformatics

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**Abstract:** The purpose of the current study was to determine if saliva contains biomarkers that can be used as diagnostic tools for Sjögren's syndrome (SjS). Twenty seven SjS patients and 27 age-matched healthy controls were recruited for these studies. Unstimulated glandular saliva was collected from the Wharton's duct using a suction device. Two μl of salvia were processed for mass spectrometry analyses on a prOTOF 2000 matrix-assisted laser desorption/ionization orthogonal time of flight (MALDI O-TOF) mass spectrometer. Raw data were analyzed using bioinformatic tools to identify biomarkers. MALDI O-TOF MS analyses of saliva samples were highly reproducible and the mass spectra generated were very rich in peptides and peptide fragments in the 750-7,500 Da range. Data analysis using bioinformatic tools resulted in several classification models being built and several biomarkers identified. One model based on 7 putative biomarkers yielded a sensitivity of 97.5%, specificity of 97.8% and an accuracy of 97.6%. One biomarker was present only in SjS samples and was identified as a proteolytic peptide originating from human basic salivary proline-rich protein 3 precursor. We conclude that salivary biomarkers detected by high-resolution mass spectrometry coupled with powerful bioinformatic tools offer the potential to serve as diagnostic/prognostic tools for SjS. (J Oral Sci 54, 61-70, 2012)

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## Introduction

Secretion of saliva in adequate quantity and quality is of paramount importance for the maintenance of oral

health (1-2). Saliva contains a variety of antimicrobial proteins and growth factors that play a crucial role in the homeostasis of the oral cavity (1-3) Saliva serves a number of different functions including lubrication and protection of oral tissues, buffering action and clearance, maintenance of tooth integrity, antibacterial activity, and taste and digestion (1-3). A decrease or lack of salivary gland secretion leads to dry mouth or xerostomia, as observed in Sjögren's syndrome (SjS).

SjS is a systemic inflammatory disease affecting primarily the lacrimal and salivary glands (4-5). It may exist as a primary disorder (primary SjS) or can be associated with other autoimmune diseases (secondary SjS) such as rheumatoid arthritis, systemic lupus erythematosus, or systemic sclerosis. The etiology of SjS, although still ill-defined, is thought to be multifactorial, involving viral, neural, genetic, and environmental factors (4,6). This fact adds an extra layer of complexity in diagnosing SjS (4-5). The prevalence of this syndrome varies widely depending on the criteria for classification, but it is estimated that between 1 and 3 million North Americans suffer from this disease (7-8). Clinically, if unmanaged, SjS can have a severe impact on the integrity of the oral cavity (4,6). SjS patients usually experience difficulty swallowing, severe and progressive tooth decay, and oral infections (particularly fungal). To date there are no treatments or cures for this disease.

SjS is considered to be the most commonly under-diagnosed autoimmune disease. It is not uncommon for there to be a delay of 5 to 8 years after symptom onset before a diagnosis is made (9-10). Furthermore, the diagnostic approach to SjS is complicated because it must include two different goals: firstly, assessment of the ocular and salivary components, and secondly, differentiation between the primary and secondary variants of the syndrome (5). Current diagnostic criteria for primary SjS include subjective (patient reported) and objective (measurable) signs of dry eye and/or dry mouth, presence of autoantibodies against the ribonucleoproteins Ro/SSA and/or La/SSB, and focal lymphocytic sialoadenitis (5). Obviously, these criteria can only detect patients with established disease when glandular destruction has already occurred. Furthermore, most of these tests either lack specificity (unstimulated salivary flow) or are invasive (lip biopsies) (11). Therefore, the development of accurate and non-invasive tests that can lead to earlier diagnosis or predict the onset of disease would be of considerable value to improve treatment and prognosis of SjS patients.

Individual markers have been used successfully in human disease diagnosis, classification, and prognosis.

However, it is now recognized that multiplexed profiling (studying multiple genes or proteins to form a marker signature) greatly improves discrimination between patient populations (i.e., diseased vs. healthy) (12-13). Mathematically, a pattern of multiple biomarkers, when compared to a single biomarker, may contain a higher level of discriminatory power (12-13). This is particularly important when studies are conducted on heterogeneous patient populations and deal with complex multifactorial diseases, such as cancer or autoimmune diseases (12-13).

Several genes, proteins, and their metabolic byproducts have been known to change during developing stages of human disease. Recently, proteomic and metabolomic marker profiles of individuals are being recognized as telltale signatures, which can provide early warning detection of disease (14-17). The latest advances in mass spectrometry coupled with the development of algorithms capable of handling the massive data generated by mass spectrometry, offer great hope for accurate and early diagnosis of human diseases.

The aim of the present studies was to determine if biomarkers that can be used for diagnosis of SjS are present in saliva. We used a combination of high-resolution mass spectrometry coupled with patented bioinformatic algorithms to analyze samples collected from well-defined SjS patients and healthy control individuals.

## Materials and Methods

### Patients

All experiments were conducted in accordance with the Declaration of Helsinki (http://wma.net). Saliva samples were collected from SjS patients and healthy controls. SjS patients were recruited from The Rubenstein Clinic at Tufts University School of Dental Medicine. Informed consent was obtained from all participants in accordance with the TUFTS-NEMC Institutional Review Board guidelines. A total of 27 SjS patients (18 with diagnosed primary SjS and 9 diagnosed with secondary SjS) and 27 healthy controls were recruited for saliva collection. SjS patients' characteristics are summarized in Table 1.

### Saliva collection

The patients were instructed not to eat, smoke, or drink for at least three hours prior to the collection of saliva. Furthermore, all collections were performed at a fixed time of the day to minimize fluctuations related to circadian rhythm in salivary secretion. The patients were seated and the floor of the mouth was isolated with sterile gauge pad. Unstimulated saliva was collected from the submandibular glands (Wharton's duct) using

Table 1  Summary of Sjögren's syndrome patients'
characteristics

|  | Primary (*n* = 18) | Secondary (*n* = 9) †¶ |
|---|---|---|
| Age* | 63 ± 10 (34-81)# | 62 ± 11 (50-81) # |
| Sex (F/M) | 14/0 | 12/1 |
| Disease duration*§ | 12 ± 8 (1-30)# | 12 ± 6 (3-20) # |
| Salivary flow rate‡ |  |  |
|    Unstimulated | 0.066 ± 0.100 | 0.017 ± 0.021 |
|    Stimulated | 0.564 ± 0.633 | 0.703 ± 0.659 |

†Five with rheumatoid arthritis, 1 with systemic lupus erythematosus
(SLE), 1 with CREST syndrome (limited scleroderma) and SLE, 1
with fibromyalgia and celiac disease and 1 with liver disease.
* Mean (years) ± S.D. § Since diagnosis. # Range (years). ‡ ml/min
(mean ± S.D.)

a suction device. Saliva samples, approximately 0.2 ml,
were transferred to a clean 1 ml microfuge tube kept
on ice and centrifuged for 20 min at 12,000 rpm in a
benchtop-refrigerated centrifuge. The samples were then
transferred to clean 1 ml microfuge tubes and kept at
-80°C until analysis.

## Sample processing and mass spectrometry

A 2 µl saliva sample volume was found to be adequate
for analyses. Sample preparation and biomarkers elution
was carried out as described by Lopez et al. (16). For
reproducibility, each sample was analyzed in triplicate
and applied to three different wells on the microtiter plate.
Samples were eluted directly from the resin ZipPlate onto
disposable MALDIchip™ targets (PerkinElmer, Boston,
MA).

Processed saliva samples were analyzed by mass
spectrometry on a prOTOF™ 2000 matrix assisted laser
desorption/ionization orthogonal time-of-flight (MALDI
O-TOF) mass spectrometer (PerkinElmer) as described
by Lopez et al. (16). Data acquisition was performed
using a mass range of 750-12,000 Daltons.

## Processing and analysis of spectral profiles

The raw mass spectral data were analyzed using
PROFILE™, a proprietary biomarker discovery
platform optimized for the sensitive and accurate identi-
fication of disease-specific signatures in high-resolution
MALDI-TOF mass spectra. This biomarker discovery
platform successfully identified biomarker signatures in
Alzheimer's disease (16), multiple sclerosis (17), and
pheochromocytoma (18).

Spectra were baseline-corrected, *m/z*-aligned, normal-
ized, de-noised, and if present, sample processing
artifacts were suppressed. The pre-processed spectra
were compared to each other and outlier spectra were

identified using a combination of spectral entropy and
Euclidean distance calculation-based methods. Spectra
that passed the outlier rejection step were used for
calculating the average spectrum for each sample. Peak
locations were identified in the spectra and peak intensi-
ties or a well-defined portion of the area under the curve
around the peak were used as input to proprietary feature
selection and model building algorithms.

During feature selection and model building, the data
set was split into a training set and a test set (two-thirds
training and one-third test), and the training set was used
to build a classification model capable of classifying
samples with optimal sensitivity and specificity. In the
feature selection step, algorithms were run to identify
features in the qualified spectral data that were clearly
different among groups. Typically, the feature selection
process identifies numerous features; however, only some
may be useful as diagnostic predictors. PROFILE™ can
select more than one type of learning algorithm and
can build multiple competing classification models. A
model consists of a particular combination of features.
The algorithms identify the minimum number of features
included in the optimal model. After the model was
built, its classification performance was evaluated on the
test set. This procedure was repeated at least 50 times
(50-fold cross-validation) and the statistical result of these
analyses yielded sensitivity and specificity figures for the
particular model. The contribution of each individual
biomarker to the model performance was evaluated
using sensitivity analysis that is the re-evaluation of the
model performance with the omission of the particular
biomarker. Performance of these models was evaluated
using external cross-validation with a test set using a
different set of samples not included in the original
model.

## Sample fractionation and Edman sequencing

Saliva samples proteins and peptides were fractionated
by high performance liquid chromatography (HPLC)
prior to protein sequencing by Edman degradation. A
200 µl aliquot of each sample was placed in an Agilent
autosampler vial and fractionated using an Agilent 1100
HPLC system (Palo Alto, CA) equipped with a quaternary
pumping system and a photodiode array (PDA) detector.
Eluted fractions were evaporated to dryness, then recon-
stituted and an aliquot of each of these fractions was
analyzed by MALDI O-TOF MS, as described above.
The mass spectral data were then interrogated for detec-
tion of candidate biomarkers. An aliquot of the remainder
of the fractions was subjected to Edman sequencing on
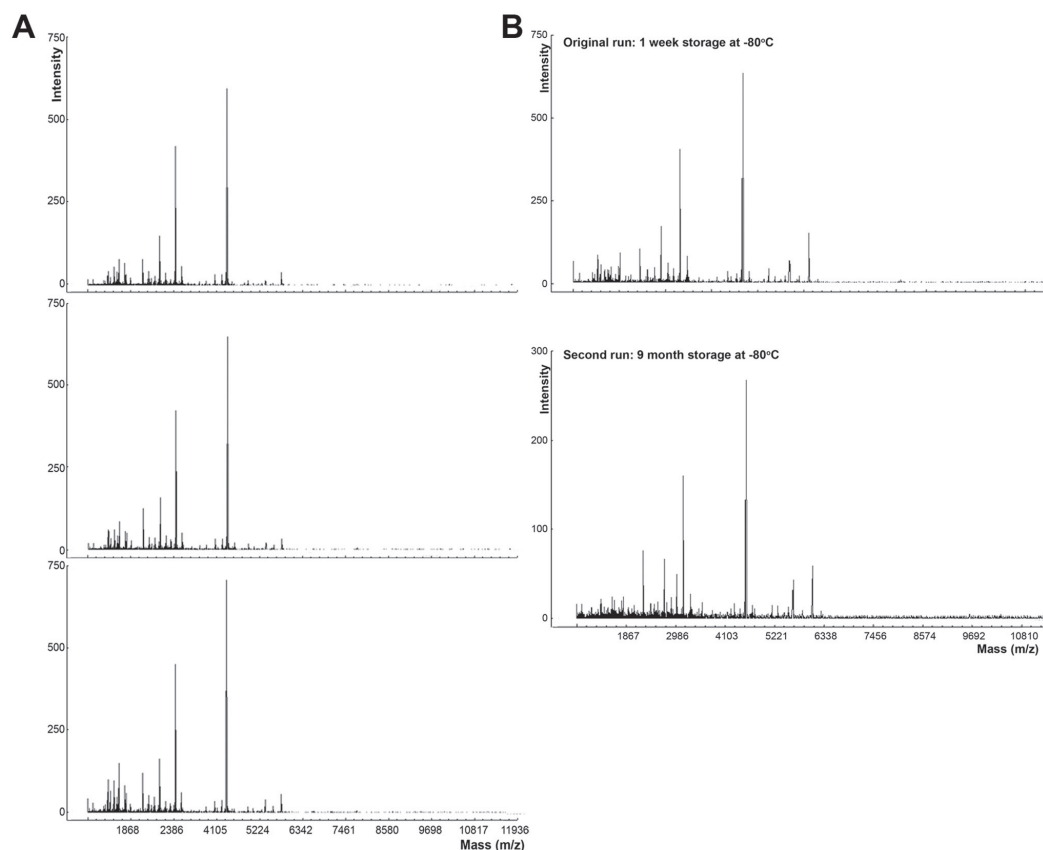an Applied BioSystems 494HT instrument.

Fig. 1 Reproducibility of sample handling and analysis. A. Shown are mass spectral profiles measured from the same saliva sample that was processed three times. B. Shown are mass spectral profiles measured from the same saliva sample that was processed after 1 week and 9 months storage at -80°C.

## Results

### Reproducibility of sample handling and analysis

For a biomarker profile to be rigorous enough to be diagnostically relevant, the profile has to be extremely reproducible (19-20). Figure 1A shows three sets of mass spectral profiles measured from the same saliva sample that was processed three times. The salivary mass spectra were very rich in peptides and peptide fragments contained mostly in the 750-7,000 $m/z$ range (Fig. 1A). Importantly, the replicate profiles were nearly identical showing the reproducibility of sample processing and data acquisition by the prOTOF MALDI-MS.

Another critical factor that can alter the mass spectral profiles and introduce errors is sample handling/storage (21). Therefore, we processed saliva samples from the same patients, 1 week following collection and storage at -80°C and then after a 9-month storage period at -80°C. Figure 1B shows representative mass spectral profiles from one subject. It is evident from Fig. 1B that both spectral profiles display similar patterns suggesting that

long-term storage (-80°C) has no (or minimal) effect on sample integrity.

Taken together, these results show that our protocol for sample preparation/storage and the data acquisition from the MALDI O-TOF MS are reproducible.

### Discovery of putative biomarkers for SjS in saliva

Saliva samples from control and SjS patients were then processed for mass spectrometry analyses. The same investigator analyzed the samples in a blind and random manner. Furthermore, to control for reproducibility, each sample was analyzed in triplicate. Figure 2 shows representative mass spectral profiles of saliva samples collected from 3 control subjects and 3 SjS patients.

A visual inspection of the mass chromatograms of three SjS' saliva samples and the mass spectral data from three control subjects indicate significant mass peak differences (Fig. 2). In all cases, the control samples and the SjS' saliva samples contained several peptides and peptide fragments that spanned the 750-10,000 $m/z$ range
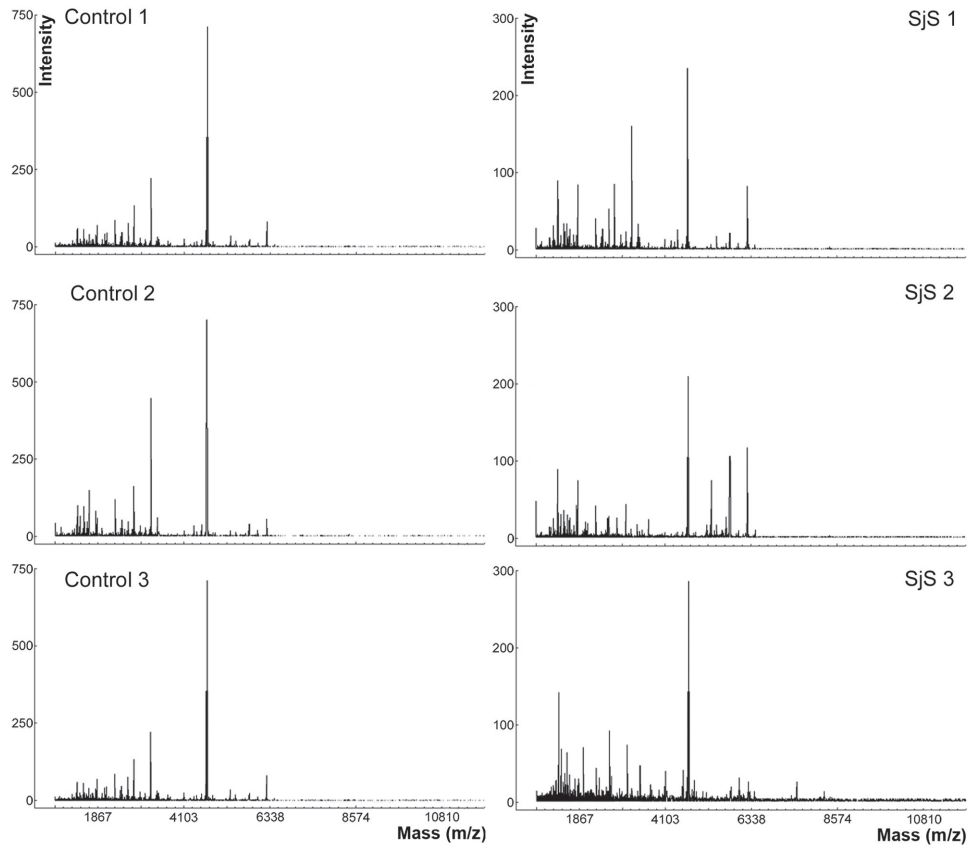
Fig. 2 Representative mass spectra of saliva samples collected from 3 control individuals and 3 SjS patients.
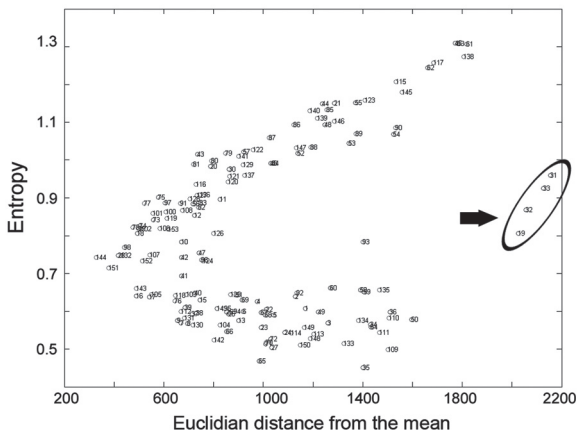


Fig. 3 Outlier detection feature of PROFILE™. Four outlier spectra were detected (arrow), 3 of which originated from the same subject.

(Fig. 2).

PROFILE™ identified 4 outlier spectra, 3 of which originated from the same subject (Fig. 3). This subject and the fourth outlier spectrum were removed before further analysis. The remaining spectra were then background-corrected and average spectra were computed.

Pattern recognition and feature selection algorithms of PROFILE™ were applied to detect spectral features distinguishing the two groups of subjects. Model performance was evaluated using 50-fold cross-validation and the model sensitivity/specificity parameters were reported. PROFILE™ successfully produced several classification models. The two best models are summarized in Tables 2 and 3. Both models are composed of 7 putative biomarkers, 3 of which are present in both models (Table 2, underlined *m/z* values). The 2 models were selected because of their high sensitivity, specificity and accuracy. Table 2 shows the mass to charge ratio (*m/z*) of the biomarkers selected for the two models generated and Table 3 shows the sensitivity, specificity and accuracy of the biomarker models.

One SjS-specific marker (*m/z* = 3803.38) showed peaks with 0.5 amu periods in the *m/z* = 3,803-3,808 range (Fig. 4). As expected, another SjS-specific marker was detected in the *m/z* = 7,606-7,618 range with 1.0 amu periods, which proves that the SjS-specific marker at *m/z* = 3,803.38 is a doubly charged ion (Fig. 4). This discovery

Table 2  Sjögren's syndrome models peptide mass generated by PROFILE™

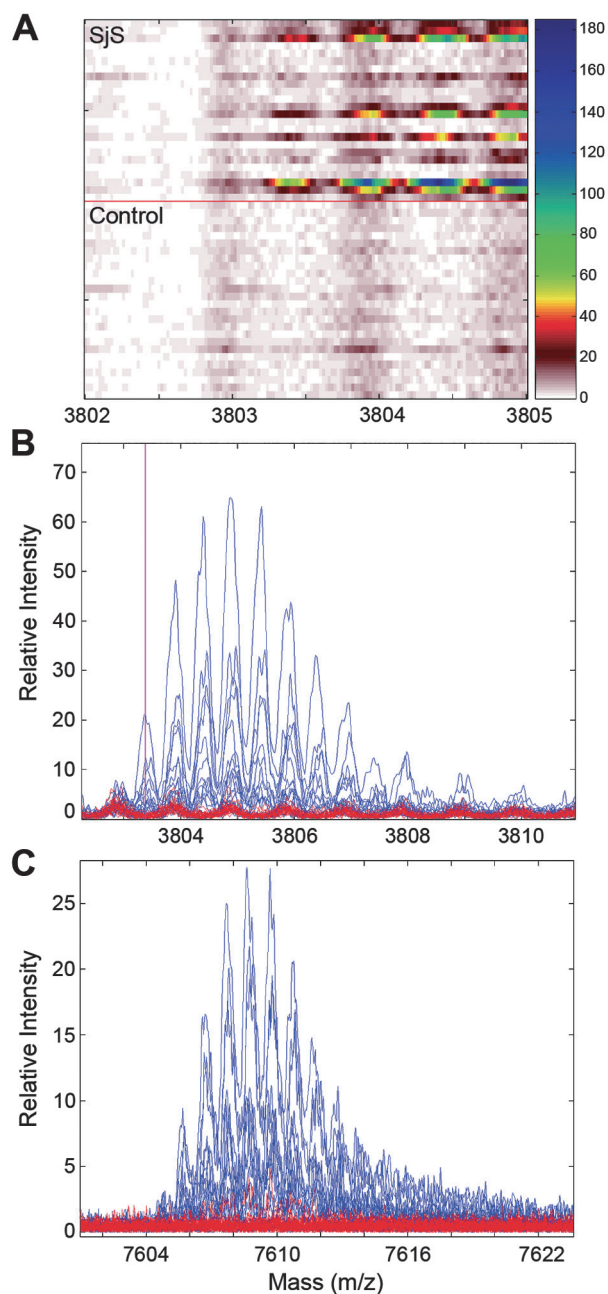| | Putative Biomarkers (*m/z*) | | | | | | |
|---|---|---|---|---|---|---|---|
| Model 1 | 902.48, | 2,407.30, | 2,912.56, | 3,655.78, | 3,803.38, | 4,281.14, | 5,942.00 |
| Model 2 | 902.48, | 1,479.76, | 2,536.16, | 3,655.78, | 3,803.38, | 4,930.28, | 5,843.94 |



Fig. 4 Heat map and spectral profile of one putative peptide biomarker. A. Heat map. B. One SjS-specific marker (*m/z* = 3,803.38) showed peaks with 0.5 amu periods in the *m/z* = 3,803-3,808 range. C. Another SjS-specific marker was detected in the *m/z* = 7,606-7,618 range with 1.0 amu periods. Blue traces, SjS samples; red traces, control samples.

Table 3  Sensitivity, specificity, and accuracy of Sjögren's syndrome models

| Model | Sensitivity | Specificity | Accuracy |
|---|---|---|---|
| 1 | 97.5% | 97.8% | 97.6% |
| 2 | 85.0% | 91.3% | 88.1% |

provides not only a novel putative biomarker for SjS but also an unexpected technical advantage. Namely, the doubly charged ion in the MALDI-TOF spectra can be detected at high-sensitivity because it is located between the background peaks where signals from singly charged ions are not detected.

These data show that saliva contain putative biomarkers that can serve to distinguish SjS patients from healthy control subjects with high sensitivity and specificity. Noteworthy is that this biomarker was detected in both primary and secondary SjS patients.

## Sample fractionation and Edman sequencing

Experiments were conducted to further characterize the molecular identity of the doubly charged ion that was only present in SjS patients' saliva samples. Saliva samples from two SjS patients were fractionated, separately, by HPLC as described in the Methods section. As shown in Fig. 5, the doubly charged SjS biomarker was detected in HPLC fraction 17. Inset A in Fig. 5 shows expanded mass spectra depicting the location of the doubly charged ion whereas the corresponding 7,606 Da peptide is depicted in inset B. Note that the singly charged ion (equivalent to protonated molecular ion) is now the largest peak in the spectrum.

The fractions (from both patients) containing the peptide associated with the doubly charged ion were then combined, concentrated and subjected to Edman sequencing. A total of 40 amino acids were then positively identified using Edman degradation sequencing (N-terminus to C-terminus) *SPPA KPQG PPPQ GGNQ PQGP PPPP GKPQ GPPP QGGN KPQG*. The partial amino acid sequence was searched against the non-redundant GenBank database and was determined to be a good match to the human basic salivary proline-rich protein 3 precursor.
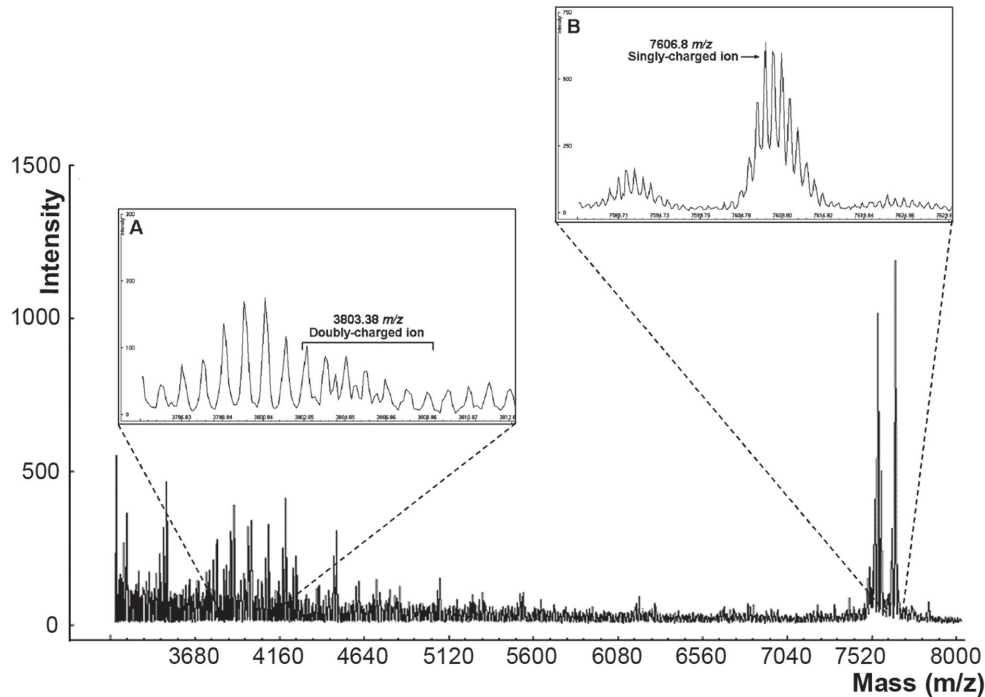
Fig. 5 MALDI mass spectra of HPLC fraction 17. Inset A. shows expanded mass spectra depicting the location of the doubly charged ion whereas the corresponding 7,606 Da peptide is depicted in inset B.

## Discussion

Saliva has been extensively used to aid in the diagnosis of several diseases including periodontal diseases, salivary gland disease and dysfunction, viral diseases, sarcoidosis, tuberculosis, lymphoma, gastric ulcers, liver dysfunction, and cancers (22-26). Several studies have reported alterations in SjS salivary electrolytes composition and/or protein concentration suggesting that saliva can be used for diagnosis of SjS (27-29). Recent proteomics studies reported that SjS saliva contain potential biomarkers (30-31). The discovery-based studies reported here confirm the potential usefulness of saliva as a diagnostic/prognostic tool for SjS.

Technologies with high throughput capabilities in genomics and proteomics led to an ability to conduct discovery-based research (32-33). Such technologies include quantitative real-time polymerase chain reaction (RT-qPCR), single-nucleotide polymorphism (SNP) analyses, gene expression arrays, and high-resolution mass spectrometry. Discovery-based research relies on the ability to analyze large quantities of data without a hypothesis, by searching for patterns (genes or proteins) that usefully discriminate between different groups of individuals (32-33).

Proteomics using high-resolution mass spectrometry holds great promise for discovery of biomarkers of human diseases from minimal amounts of biological fluids (34-35). This technology offers numerous advantages: ease of operation, high-throughput capability, requirement of minimal volume of biological fluids, high sensitivity, and high resolution for low molecular weight proteins and peptides. Surface enhanced laser desorption/ionization (SELDI) mass spectrometry is a variant of MALDI that uses commercially prepared chromatographic chip array surfaces to separate proteins in the sample (36-37). Although many different chip arrays (hydrophobic, anionic, cationic, or metal binding) may be used on one sample, the choice of chip array can require some prior knowledge of the changes that may take place between control and test or disease subjects, in order to select the correct chip array to yield proteins of interest (35). Furthermore, compared to MALDI-MS, SELDI-MS have relatively poor resolution.

Using SELDI-MS coupled to two-dimensional difference gel electrophoresis (2D-DIGE), Ryu et al. identified ten putative salivary biomarkers in the SjS group that differed from control individuals (30). Seven of the biomarkers were already known whereas three were novel (30). Similarly, using MALDI-MS, Hu et al. reported that the expression of 16 peptides was different in primary SjS and controls, with 10 of the peptides being

overexpressed and 6 suppressed in SjS patients (31).

The issue of molecular characterization of mass spectrometry-based biomarker discovery is still hotly debated in the scientific community. There are two schools of thought: one that advocates for fully identified biomarker molecule (38) and the other favoring discriminating "ion patterns"(12). It is argued that without molecular characterization of the biomarkers identified by mass spectrometry, this methodology will remain empirical and the results will be difficult to validate, reproduce, and standardize (38). As a counterargument, it is possible that using high-resolution mass spectrometry (such as MALDI-TOF), "each ion is assigned a mass with such precision and accuracy that the accurate mass tag becomes an identifier" (12).

In this report, we used mass spectrometry and bioinformatic search algorithms to identify a 7,606 Da biomarker peptide unique to the salivary samples from the SjS subjects. The presence of this unique ion was made even more notable by the observation that the doubly charged ion of the same peptide was also present in the SjS subjects. Using additional analytical tools, we were able to determine a partial peptide sequence for the peptide. When the sequence was searched against the non-redundant GenBank public database, we determined a good match to the human basic salivary proline-rich protein 3 precursor (PRP3). PRPs are divided into acidic, basic, and glycosylated PRPs. Acidic PRPs bind calcium and inhibit hydroxyapatite crystal growth thus contributing to calcium homeostasis in the mouth and the acquired enamel pellicle of erupted teeth (39). PRPs are encoded by six genes with each gene having several alleles giving rise to ~20 secreted proteins showing complex patterns of polymorphism (40). PRPs are synthesized as precursor proteins and undergo proteolysis as well as posttranslational modifications giving rise to basic PRPs and glycosylated PRPs. The proteases responsible for the processing of the precursor proteins are called convertases and several studies have shown that furin (known to be expressed in salivary glands) is one of these convertases (41).

The first ten amino acid of the 7,606 Da biomarker peptide, SPPAKPQGPP, conform to a furin recognition sequence (XPPXXPXXP) which was shown to be present in precursor PRPs and to be cleaved by furin, *in vitro* and *in vivo* (41). It is therefore likely that this biomarker peptide is a proteolytic product from one of the salivary PRPs. Furthermore, the fact that this peptide was only present in saliva from SjS patients, suggests that the activity of the convertase(s) responsible for its generation is high in SjS patients' salivary glands. It is likely

that this biomarker is one of those currently detected in one or both models and awaiting identification.

The biomarkers identified in this study were present in samples from primary as well as secondary SjS patients. It is not clear if these biomarkers were present before disease manifestation or are a consequence of the salivary damage associated with the disease. We found no association between the secondary disease, age, smoking history and the presence of these biomarkers. Furthermore, sialogogues (Salagen or Evoxac), antihistamine, and antidepressants were the most commonly used medications in our study population and their use was not correlated with any of the reported biomarker.

In summary, using a discovery-based approach, we report that saliva contains putative biomarkers capable of distinguishing SjS patients from healthy control individuals with high sensitivity and specificity. The next step will be to validate these biomarkers in an independent and larger SjS patient cohort and control individuals. Furthermore, patients with xerostomia but no SjS and patients with another autoimmune disease but no xerostomia should also be included in the validation studies. As the putative biomarker was present in saliva of both primary and secondary SjS patients, another study should address the question whether the same biomarker is present in autoimmune disorders without lacrimal and salivary gland involvement.

## Acknowledgments

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