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ORIGINAL ARTICLE

Different proteomic protein patterns in saliva of Sjögren's syndrome patients

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OBJECTIVE: To investigate the salivary protein profile in patients with Sjögren's syndrome (SS), and healthy control subjects.
MATERIALS AND METHODS: Unstimulated whole saliva samples were collected from 16 age-matched females; eight healthy subjects and eight patients diagnosed with SS (six primary SS, one incomplete SS and one primary SS associated with B cell lymphoma). Proteins were extracted and separated individually by 2D sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Selected protein spots of interest were analysed by electrospray ionization – tandem mass spectrometry. Obtained data were searched against the Swiss-Prot and NCBI nonredundant protein databases using Mascot software.
RESULTS: Two groups of patterns of protein expression were observed in the eight SS patients: a major group (six patients) with significant expression differences from the healthy subjects and the second group (two patients) with a pattern similar to the eight healthy subjects.
CONCLUSION: In this preliminary study, protein expression differences were found between SS patients and healthy subjects. Individual analysis of SS patients exhibited two patterns of protein expression with no direct relation to the clinical, serological or histological severity of disease. This study emphasizes the difficulty of the present proteomic knowledge to diagnose and monitor the sequel of SS development.
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Keywords: proteomics; biomarkers; Sjögren's syndrome; saliva; 2-DE

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Introduction

Sjögrens' syndrome (SS) is an autoimmune exocrinopathy with a clear female predilection characterized by lymphocytic infiltration of the affected glands. The disease targets salivary and lacrimal glands (Fox, 2007). Consequently, patients suffer from severe dryness of the mouth and eyes (Fox, 2007). As no definitive clinical or serological marker for identification of SS is available, different classification criteria sets have been suggested; one of the most accepted currently is the one recommended by the American–European consensus group (Vitali *et al*, 2002). Moreover, no serological markers such as the anti SS-A (Ro), anti SS-B (La), calpastatin and alphafodrin or the intensity of lymphocyte infiltration into the labial salivary glands (focus score) proportionally correspond to the severity and stages of the SS (Goëb *et al*, 2007).

As salivary glands play a major component in the SS sequel, several studies have explored markers in saliva as an early diagnosis methodology (Castro *et al*, 2003; Kalk *et al*, 2002; Peluso *et al*, 2007). In the rapid progressive field of disease diagnostics based on salivary biomarkers, it is becoming clear that a combination pattern of several biomarkers but not a single one may define a specific disease (Wong, 2006). Having a biomarker set specifically for SS will assist in the clinical diagnosis and the severity status of SS and furthermore may contribute to the understanding of the molecular mechanism of this syndrome.

In previous studies exploring saliva from SS patients, it has been shown that protein expression differences exist in comparison with healthy individuals (Peluso *et al*, 2007; Ryu *et al*, 2006; Giusti *et al*, 2007a). Most of the previous reports used pool saliva from SS patients (Ryu *et al*, 2006; Hu *et al*, 2007). In this study, we compared proteomic analysis on individual basis of saliva from female SS patients with age-matched healthy volunteers. We have found a unique proteomic map pattern in six of the eight patients. Interestingly, the other two SS patients demonstrated a proteomic pattern resembling the control group. Comparison of protein expression profiles with those reported for diffuse systemic sclerosis (SSc) (Giusti *et al*, 2007b) shows large similarities. These observations indicate the challenges

in using salivary proteomics to diagnose, assess and monitor the sequence of SS state and severity.

Materials and methods

Patients, healthy volunteers and saliva collection

The whole saliva accumulation protocol was approved by the Ethical Committee of Hadassah Medical Center, Jerusalem, Israel. Unstimulated whole saliva flow was collected for 10 min using the spitting method into a precalibrated tube from 16 female individuals; eight healthy age-matched subjects (mean age ± s.d., 56.6 ± 9.3 years), and eight SS patients (mean age ± s.d., 51.5 ± 4.8 years). Inclusion criteria for healthy subjects consisted of volunteers not taking any medication including oral contraceptives, with no complaints of oral or ocular dryness and no oral mucosal diseases. The diagnosis of primary SS was based on the American–European consensus criteria (Vitali *et al*, 2002). Six out of the eight patients (patients' no. 1–6) fulfilled the American–European consensus criteria. Exclusion criteria included past head and neck radiation treatment, hepatitis C infection, acquired immunodeficiency disease, sarcoidosis, graft *vs* host disease, use of anticholinergic drugs, smoking habits or other well-defined connective tissue diseases. Patient no. 7 did not fulfill all the necessary criteria for diagnosis for SS and patient no. 8 was primarily diagnosed with primary SS and developed follicular lymphoma. Patient glandular signs and symptoms, extraglandular manifestations and laboratory findings are listed in Table 1. All volunteers refrained from eating, drinking and brushing their teeth 1 h prior to saliva collection. Patients did not take their medications, including sialogogues, prior to the saliva collection.

Volunteers were asked to rest for 10 min before saliva collection, sitting in an upright position and in a quiet room and were asked not to speak or leave the room until saliva collection ended. Saliva samples were immediately kept on ice and thereafter were centrifuged at 14 000 **g** for 20 min at 4°C to remove insoluble materials, cell debris and food remnants. The supernatant of each sample was collected and protein concentration was determined using the Bio-Rad protein assay according to Bradford (Bradford *et al*, 1976) (Bio-Rad, Hercules, CA, USA). Supernatants were frozen at −70°C and lyophilized overnight. Sediments were dissolved in 7 M urea, 2 M thiourea and 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS) and stored at −20°C until analysis.

2-DE

For analytical gels, samples of 100 μg protein were subjected to re-hydration followed by isoelectrofocusing in 18 cm long, pH 3–10 NL Immobiline DryStrips gels (Amersham Biosciences, Uppsala, Sweden) as we previously described (Reichenberg *et al*, 2005). Re-hydration was carried out for 16 h in 7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol and 0.5% carrier ampholytes (bio-lyte 40% 3/10 Bio-Rad) and a trace of bromophenol blue, with a constant voltage of 50 V at 20°C using a

Table 1 Patient characteristics and laboratory findings

Patient	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Glandular symptoms								
Xerostomia	+	+	+	+	+	+	+	+
Xerphthalmia	+	+	+	+	+	+	+	+
Glandular signs								
Recurrent parotitis	+	–	+	–	–	–	–	+
Unstimulated whole salivary flow (≤ 0.1 ml/min)	+	+	+	+	+	+	+	+
Schirmer I test (≤ 5 mm/5 min)	+	+	+	+	+	+	+	+
LSG score	N.D.	N.D.	1	N.D.	2	N.D.	0	4
Arthalgia	+	+	+	+	+	+	+	+
Myalgia	+	+	+	–	+	–	–	+
Extraglandular manifestations								
Raynaud's phenomenon	–	+	–	–	–	–	–	–
Peripheral neuropathy	–	–	–	–	–	–	–	–
Renal involvement	–	–	–	–	–	–	–	–
Laboratory findings								
High ESR	+	+	+	+	+	+	+	+
High RF	+	+	+	+	–	+	–	–
High CRP	–	–	–	–	–	–	–	+
Anaemia	+	+	+	–	–	+	–	–
Leucocytopenia	–	+	–	+	–	+	–	+
ANA	+	+	+	+	+	+	–	+
Anti-Ro/SS-A antibodies	+	+	+	+	+	+	–	+
Anti-La/SS-B antibodies	+	+	–	+	+	–	–	–
Low C3	–	–	–	N.D.	–	–	–	–
Low C4	–	–	–	N.D.	–	–	–	–

Patients no. 1–6 were diagnosed with primary Sjögren's syndrome (SS). Patient no. 7 did not fulfilled all the required criteria and patient 8 was diagnosed with primary SS that afterwards developed lymphoma. LSG, labial salivary gland biopsy; ESR, Erythrocyte sedimentation rate; RF, rheumatic factor; CRP, C-reactive protein; ANA, anti nuclear antibodies; SS, Sjögren's syndrome; N.D., not determined.

Protean IEF Cell (Bio-Rad). The voltage was then gradually increased to 10 000 V at 20°C and the samples were focused additionally for 8 h. To prepare the gel strips for separation in the second dimension, the strips were soaked twice for 15 min in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 0.05 M Tris–HCl pH 6.8, 2% dithiothreitol) and then (second equilibration) in SDS–PAGE equilibration buffer solution, but the dithiothreitol reagent was substituted by 2.5% iodoacetamide. For the second dimension, strips were embedded in 0.5% agarose containing trace bromophenol blue and loaded onto hinged spacer plates (20 cm × 20.5 cm; Bio-Rad) using 9.5–16.5% SDS polyacrylamide gradient gel. Electrophoresis was conducted simultaneously for all 12 samples on the same running and staining apparatus at a constant current of 30 mA per gel at 10°C until the bromophenol blue dye front band reached the bottom of the gel. For protein detection, the 12 gels were stained with ammoniacal silver nitrate using the Dodeca Stainer Shaker (Bio-Rad).

Imaging and statistical analysis

Gels were scanned using a computer GS-800 calibrated densitometer (Bio-Rad) and protein spots were detected and quantified using PDQuest software V 6.2.0 (Bio-Rad). Several limitations exist in 2D gels analysis because of gel to gel variation, and also variability in the staining method. To overcome these drawbacks, all the sample gels went through the same conditions simultaneously over the first and second dimension. Normalization was performed to semi-quantify spot intensities and to minimize staining variation between gels. The scanned gels were divided into two groups of eight healthy and eight SS patients. A Student's *t*-test was performed, to evaluate protein expression levels according to the two groups, using GraphPad Prism software (La Jolla, CA, USA). $P < 0.05$ was considered significant for these preliminary exploratory analyses. The proteins, which differently expressed with statistical differences, were selected and identified. The mean and standard deviation were computed for these proteins.

Mass spectrometry identification and database searching

For mass spectrometric identification, a 2D SDS–PAGE containing 520 μg protein was prepared and fixed in 50% ethanol, 12% acetic acid for 2 h. Proteins were visualized by staining for 15 h with Coomassie Brilliant Blue G 250 (Fluka, Buchs, Switzerland), followed by 20% ethanol destaining. Electrophoretically separated protein spots, selected by matching them with the silver-stained gels, were excised from two dimensional gels, washed with 100 mM ammonium bicarbonate, pH 8.0, and were reduced with 45 mM dithiothreitol for 30 min at 60TC for in-gel digestion. Alkylation was performed by adding 100 mM iodoacetoamide in the dark for 30 min and the solution was discarded (Rosenfeld *et al*, 1992). Gel pieces were shrunk by 50% acetonitrile, 50% ammonium bicarbonate 100 mM, dried with 100% acetonitrile followed by a Speed-Vac. Gel pieces were re-hydrated with a solution of sequencing grade trypsin (Promega, Madison, WI, USA), 10 μg ml^{-1} in 25 mM ammonium bicarbonate and the digestion was carried out for 16–20 h at 37TC. Peptides were extracted from the gel thrice by adding two volumes of solution of 60% acetonitrile/1% formic acid. The extracts were combined and reduced to a final volume of 5–10 μl. The extracts were desalted using the ZipTipC18 (Millipore, Bedford, MA, USA) cleanup step that was performed according to the manufacturer's protocol. The ZipTip eluent was injected onto a Qtof2 (Micromass, Manchester, UK) equipped with a nanospray capillary (Wilm and Mann, 1996) and analysed by electrospray ionization tandem mass spectrometry (ESI-MS/MS).

Protein identification was accomplished by isolating, within the mass spectrometer, a peptide ion population with a single mass-to-charge ratio (m/z), fragmenting this population, and measuring the masses of the peptide fragment ions. Data analysis was performed using the biolynx package (Micromass). The experimentally determined peptide fragment ion masses were used to search a theoretical fragment ion mass database generated in a silico digestion and fragmentation of all proteins in the Swiss-Prot and NCBI data banks (http://www.expasy.org, http://www.ncbi.nlm.nih.gov respectively) using the Mascot package (Matrix Science, London, UK). The experimental fragment ion masses were matched with theoretical peptide fragment ion masses within the window of experimental mass measurement accuracy of ± 0.3 Da.

Results

2D gel map of SS and healthy subjects whole saliva proteins

About 300 protein spots were detected in each gel when using ammoniacal silver nitrate staining. Comparison of results of protein expression in SS patients and healthy subjects revealed a subdivision of SS patients into two different groups: the gels of SS patients no. 1–4, 7, 8 (termed SS-1) demonstrated substantial pattern differences of protein spots compared to healthy subjects, whereas gels of SS patients no. 5, 6 (termed SS-2) revealed an appearance much similar to the eight healthy subjects. Figure 1 is representative maps of a patient of SS-1 group, a patient of SS-2 group and a healthy subject. Marked regions demonstrate the observed differences between the two gels. Regions I and II in Figure 1 depict protein spots not detected in regions III and IV (Figure 1).

Identification of representative salivary proteins

We selected protein spots (1–29) for MS identification. Our selection criteria were based on: (i) previously published proteomic maps (Giusti *et al*, 2007a; Hu *et al*, 2005; Vitorino *et al*, 2004; Huang, 2004) and (ii) Identifying expression changes between the healthy and the two SS patient groups. Table 2 shows the MS identification of spots 1–29. Spots no. 4 (serum albumin), 19 (salivary alphaamylase), 9 (beta-actin), 12

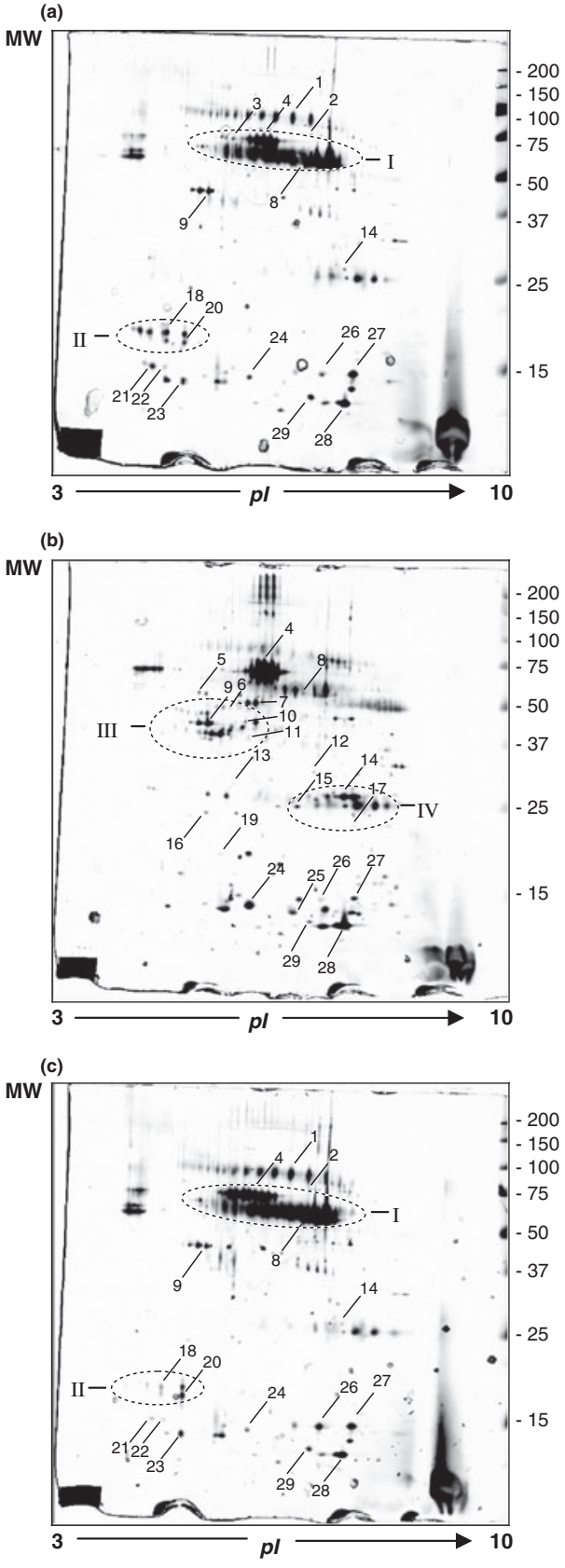

Figure 1 Representative 2D gel maps of a healthy subject (**a**), SS-1 (**b**) and a SS-2 patient (**c**). Regions of observed differences in spot protein expression are encircled with a line. Spots identified are numbered

(alpha enolase) and 18 (Lipocalin-1, also called Von Ebner gland protein) are in accord with the previously published maps.

Identification of over- and under-expressed proteins in SS

To explore protein expression in SS and healthy subjects and to explore further the subdivision among the SS patients groups, we compared 2D gels of healthy subjects with each of the SS groups, as well as between the two SS groups.

Table 3 summarizes the over- and under-expressed proteins in SS-1 group compared with the healthy group. A *t*-test statistical analysis of proteins expressed significantly different ($P < 0.05$) in the SS-1 group compared with the healthy group revealed 17 protein spots, 10 over-expressed (spots no. 6, 7, 10, 11, 14, 15, 16, 17, 24 and 28) and seven under-expressed (spots no. 1, 2, 5, 8, 20, 23 and 26). Among these, four protein spots (spots no. 2, 20, 23 and 26) were not identified because of low sample concentration. Table 3 also represents the over-expressed proteins in the SS-2 group compared to the healthy group. Statistical analysis of proteins that were expressed significantly different ($P < 0.05$) in the SS-2 group compared to the healthy group revealed two protein spots (spots no. 3, 28), both over-expressed. Only one protein was identified for the same reasons mentioned above.

Statistical analysis of proteins that were significantly different in the SS-1 group compared to the SS-2 group (Table 3) revealed four protein spots (spots no. 1, 3, 8 and 26), all under expressed. Figure 2 demonstrates the expression changes of each protein, of the healthy, SS-1 and SS-2 groups.

Discussion

In the present study, we analysed whole saliva on individual basis from six patients diagnosed with primary SS according to the American–European Consensus Group criteria (Vitali *et al*, 2002) as well as one symptomatic patient not fulfilling the criteria completely and one who had developed follicular lymphoma. Our goal was to explore individual proteomic patterns of SS patients compared with healthy subjects. From the eight patients, 2D gels of six patients (SS-1 group) demonstrated a different protein expression pattern compared to the healthy group. This pattern was similar to that of the recently reported studies (Ryu *et al*, 2006; Giusti *et al*, 2007a; Hu *et al*, 2007; Giusti *et al*, 2007b).

Interestingly, another pattern resembling the healthy group was found in the other two patients. This finding demonstrates the need to examine proteomic patterns both individually and in pooling during this developmental stage of salivary diagnostics. Previous reports used pooled saliva samples for analysis (Ryu *et al*, 2006; Hu *et al*, 2007); therefore, it was not possible to explore individual differences to detect subgroup patterns as was done in this study. For exploratory analyses, we chose to pool SS patients by proteomic patterns, independent of the level of salivary gland infiltration (Table 1). As these analyses were exploratory, we accepted an alpha error of

Table 2 Identification representative salivary proteins, and the over and under expressed proteins found in the saliva of the healthy and the SS-1 groups

Spot no.	*Protein name*	*Swiss-Prot AC number*	*Theoretical pI/MW*	*Sequence coverage (%)*	*Ion score*
1	Polymeric-immunoglobulin receptor	P01833	5.59/83 313	5	92
2	N.D.	–	–	–	–
3	N.D.	–	–	–	–
4	Serum albumin	P02768	5.92/69 366	30	729
5	Vitamin D-binding protein	P02774	5.40/52 963	9	63
6	Keratin, type I cytoskeletal 10	P13645	5.13/59 518	23	636
	Keratin, type II cytoskeletal 1	P04264	8.16/66 017	13	380
7	Serum albumin	P02768	6.47/57 767	33	624
8	Salivary alpha-amylase	P04745	6.47/57 767	51	1066
9	Beta-actin	P60709	5.29/41 736	37	433
10	Actin, alpha skeletal muscle (alpha-actin-1)	P68133	5.23/42 051	9	77
11	Keratin, type I cytoskeletal 10	P13645	5.13/59 518	7	105
	Fibrinogen beta chain	P02675	8.54/55 928	3	85
	Serum albumin	P02768	6.47/57 767	6	96
12	Alpha-enolase	P06733	7.01/47 168	24	317
13	Keratin, type II cytoskeletal 1	P04264	8.16/66 017	5	96
	Keratin, type I cytoskeletal 10	P13645	5.13/59 518	9	89
14	Serum albumin	P02768	5.92/69 366	19	498
15	Serum albumin	P02768	5.92/69 366	20	462
16	Beta-actin	P60709	5.29/41 736	32	618
17	Ig gamma-1 chain C region	P01857	8.46/36 105	27	328
18	Lipocalin-1 (Von Ebner gland protein)	P31025	5.39/19 250	12	63
19	Salivary alpha-amylase	P04745	6.47/57 767	11	61
20	N.D.	–	–	–	–
21	Keratin, type II cytoskeletal 1	P04264	8.16/66 017	10	235
22	N.D.	–	–	–	–
23	N.D.	–	–	–	–
24	S100 calcium-binding protein A9 (calgranulin-B)	P06702	5.71/13 241	37	175
25	S100 calcium-binding protein A7 (Psoriasin)	P31151	6.27/11 456	53	254
26	N.D.	–	–	–	–
27	Serum albumin	P02768	5.92/69 366	7	182
	S100 calcium-binding protein A9 (calgranulin-B)	P06702	5.71/13 241	26	53
28	S100 calcium-binding protein A8 (calgranulin-A)	P05109	6.51/10 834	49	312
	Phenylalanyl-tRNA synthetase alpha chain	Q9Y285	7.31/57 563	2	60
29	Beta-2-microglobulin	P61769	6.06/13 714	26	116
	Keratin, type I cytoskeletal 10	P13645	5.13/59 518	7	83
	Keratin, type II cytoskeletal 1	P04264	8.16/66 017	5	81

Theoretical *pI* and *MW* values are according to the Swiss-Prot protein database. Ion score was calculated as 10*Log(P), where *P* was the probability of the observed match to be a random event. N.D., Non-determined.

Table 3 Statistical analysis of proteins with significantly different expression between groups

Spot no.	*Protein name*	P-*value*	*Folds in SjS-1*
SS-1 group *vs* healthy group			
1	Polymeric-immunoglobulin receptor	< 0.05	↓ 21.8
2	N.D.	< 0.05	↓ 28.5
5	Vitamin D-binding protein	< 0.05	↓ 8.2
6	Keratin, type I cytoskeletal 10 and keratin, type II cytoskeletal 1	< 0.05	↑ 6.4
7	Serum albumin	< 0.05	↑ 231.4
8	Salivary alpha-amylase	< 0.01	↓ 9.1
10	Actin, alpha skeletal muscle (alpha-actin-1)	< 0.01	↑ 38.4
11	Keratin, type I cytoskeletal 10 and fibrinogen beta chain and serum albumin	< 0.001	↑ 10
14	Serum albumin	< 0.05	↑ 7.8
15	Serum albumin	< 0.05	↑ 105.5
16	Beta-actin	< 0.05	↑ 7
17	Ig gamma-1 chain C region	< 0.05	↑ 12
20	N.D.	< 0.01	↓
23	N.D.	< 0.05	↓ 31.9
24	S100 calcium-binding protein A9 calgranulin-B	< 0.05	↑ 5.3
26	N.D.	< 0.05	↓ 20.1
28	S100 calcium-binding protein A8 calgranulin-A and phenylalanyl-tRNA synthetase alpha chain	< 0.05	↑ 2.8
SS-2 group *vs* healthy group			Folds in SjS-2
3	N.D.	< 0.001	↑ 5.9
28	S100 calcium-binding protein A8 calgranulin-A and phenylalanyl-tRNA synthetase alpha chain	< 0.05	↑ 2.2
SS-1 group *vs* SS-2 group			Folds in SjS-1
1	Polymeric-immunoglobulin receptor	< 0.05	↓ 30.6
3	N.D.	< 0.001	↓ 25.1
8	Salivary alpha-amylase	< 0.01	↓ 14.2
26	N.D.	< 0.01	↓ 6.5

P-value was calculated according to the student's *t*-test.

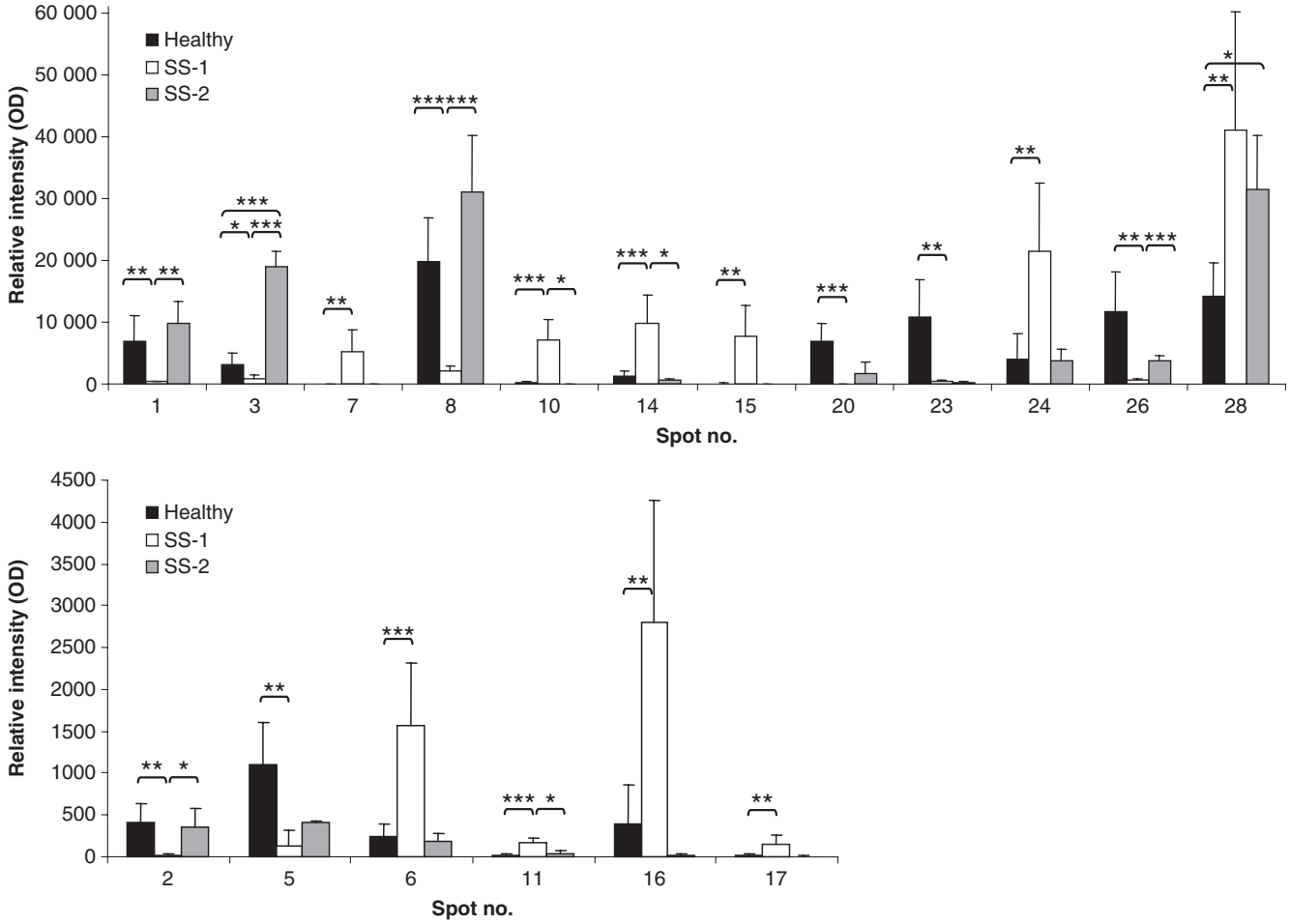

Figure 2 Differently expressed proteins found in the saliva of SS-1, SS-2 and healthy groups. Each bar represents the mean ± SD of the mean of each spot. Significant differences were calculated according to student *t*-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

0.05%, even though multiple comparisons were made and the SS subgroups were very small.

We detected a significant reduction in polymeric-immunoglobulin receptor (pIgR; spot no. 1) in the SS-1 group compared with the healthy group (Figure 2). pIgR binds polymeric IgA and IgM at the basolateral surface of epithelial cells. The complex is then transported across the cell to be secreted at the apical surface. This reduction in expression can be explained by the decrease in acinar cell function in SS patients. However, previous studies including an SS study have reported up-regulation of pIgR in several chronic inflammatory mucosal diseases (Schjerven *et al*, 2000). Ryu *et al*, (2006) also showed an increase in pIgR of parotid saliva of SS patients. This inconsistency with our study could be explained by the different salivary gland sources of saliva collection, i.e. in our study, unstimulated whole saliva was collected representing the entire salivary gland secretion.

Vitamin D-binding protein (DBP, spot no. 5) is a multifunctional protein found in plasma, ascitic fluid, cerebrospinal fluid, urine and also in saliva. It is also found on the surface of many cell types; DBP associates with membrane-bound immunoglobulin on the surface of B-lymphocytes and with the IgG Fc receptor on membranes of T-lymphocytes. We found significantly decreased DBP in SS-1 group subjects compared to the healthy subjects. Muller *et al*, (1990) found abnormal vitamin D3 metabolism in patients with primary SS. They have suggested that vitamin D metabolism changes in patients with systemic autoimmune diseases. The serum concentration of vitamin D (25-OHD3) was found to be significantly below the normal in primary SS patients. This observation is not clear and it is speculated that vitamin D possesses an immunoregulatory role.

Keratin type I cytoskeletal 10 (spots no. 6, 11) and keratin type II cytoskeletal 1 (spot no. 6) are both members of the cytokeratin proteins. These proteins are found in the cytoskeleton of epithelial cells, such as acinar cells and have been reported to be found in saliva (Xie *et al*, 2005). The significant elevation we found in these proteins in the SS-1 group is possibly because of acinar cell damage and leakage of these keratins content into saliva.

Albumin, which is the main protein of plasma, is known to infiltrate saliva from the enclosed capillary bed without further secretion into the oral cavity. Several studies have reported an increase in albumin level in SS patients (Van der Reijden *et al*, 1996; Stuchell

et al, 1984; Sreebny and Zhu, 1996). This is consistent with our data demonstrating a significant increase in albumin levels (spots no. 7, 11) in the SS-1 group compared with the healthy. We also found a significant reduction in salivary alpha-amylase (spot no. 8) in the SS-1 group compared with the SS-2 and the healthy groups. It was suggested that this decrease was caused by atrophy of salivary gland and damage of acinar parenchyma (Ryu *et al*, 2006; Giusti *et al*, 2007a).

Calgranulin-A and calgranulin-B (spots no. 28 and 24 respectively) are members of the s-100 calcium-binding protein family. Calgranulin-A was found to have significantly increased in the SS-1 and SS-2 groups compared to the healthy group, whereas calgranulin-B had significantly increased only in the SS-1 group. These proteins are expressed by macrophages in tissue inflammation processes. Although these proteins are not specific for SS (Sweet *et al*, 2001; Hu *et al*, 2004; Ott *et al*, 2003), their combination with other differentially expressed proteins may assist in finding salivary biomarker patterns for SS.

Proteins that were increased in the SS-1 group compared to healthy controls were alpha and beta actin (spots no. 10, 16), proteins involved in various types of cell motility that are ubiquitously expressed in all eukaryotic cells, and phenylalanyl-tRNA synthetase alpha chain, an enzyme that catalyses the attachment of an amino acid to its cognate transfer RNA molecule in a highly specific two-step reaction. The functions of these proteins in whole saliva remain unknown.

The prolactin-inducible protein (PIP) was not identified in this work; however, spot no. 20 was suspected of being PIP according to previously published salivary protein maps (Giusti et al, 2007a; Hu *et al*, 2005, Vitorino *et al*, 2002; Huang, 2004). Spot no. 20 was significantly decreased in the SS-1 group compared with the healthy group. This secretory protein has several functions including in enamel pellicle development, in innate immunity and in mucosal defender (Mirels *et al*, 1998).

Spot no. 26 was suspected of representing cystatin SN precursor. We found this protein to be significantly decreased in the SS-1 group compared to the healthy and the SS-2 groups. Cystatin SN is a member of the cystatin superfamily of cysteine proteinase inhibitors. These protein families take part in inflammatory and immune responses by protecting against tissue damage caused by proteinases (Schjerven *et al*, 2000). It is speculated that under-expression of cystatin SN in SS-1 patients is related to loss of balance between proteases and their inhibitors consequently reflecting the salivary gland impaired function in SS (Giusti *et al*, 2007a).

The protein patterns detected in these eight patients possibly demonstrate a progressive inflammatory process in SS salivary glands, as the two SS-2 patients had fewer inflammatory proteins. Moreover, the SS-2 group proteomic map showed resemblance to the healthy group map, even though the patients in this group are well matched with the worldwide accepted diagnostic criteria of primary SS patients (Vitali *et al*, 2002). This discrepancy raises interesting questions: (1) Can SS patients be diagnosed solely based on the proteomic analysis of saliva? (2) Will different subpopulations of SS patients have different proteomic patterns? and (3) Can progressive stages of SS salivary gland pathology be detected with salivary proteomic patterns?

Another factor that may limit the diagnostic potential of salivary proteomic patterns for SS is lack of specificity. A recent study exploring salivary proteins from patients with SSc (Giusti *et al*, 2007b) found five of the nine proteins (corresponding to 13 protein spots) differentially expressed in the whole saliva of SSc patients compared to healthy subjects, have been identified in whole saliva of SS patients in the present study and/or in previous reports. These include calgranulin B [this study, (Giusti *et al*, 2007a; Hu *et al*, 2007), beta 2-microglobulin (Hu *et al*, 2007), calgranulin A (this study, Hu *et al*, 2007), cyclophilin A (Giusti *et al*, 2007a) and psoriasin (Hu *et al*, 2007)]. This suggests that these whole proteins reflect a rheumatic inflammatory process that is not unique to SS. Further comparisons should be made between salivary proteins expression of other mixed connective tissue diseases to reveal and refine the specificity of salivary protein biomarkers.

Changes in the saliva representation of high abundance proteins such as alpha amylase (or others) may be indicative of tissue damage, but rarely provide a specific marker of disease. Lower abundance proteins such as cytokines that are present at the pictograms level may offer markers for clinical diagnostics, but require sensitive detection tools. Therefore, one additional approach that should be considered is the use of a high abundant proteins removing device for amylase and albumin. Together these two protein groups consist of about 75% of salivary proteins. Removing them, as done for serum albumin and immunoglobulins, may improve both gel resolution for low abundant proteins and unmasking the 60 kDa area occupied by the high abundant amylase and albumin proteins. Another approach was made using gene chip followed by real time PCR analysis of whole saliva (Hu *et al*, 2007). This method revealed factors such as interferon (IFN) and IFN-inducible protein G1P2 specifically expressed in SS patients. These are low abundant proteins and could not be demonstrated by the regular 2-dimensional electrophoresis (2-DE) used indicating the need for pretreatment of whole saliva to remove high abundant protein fractions.

In conclusion, in this preliminary individual saliva sample analysis, study of SS patients exhibited two patterns of protein expression with no direct correlation to the clinical manifestations of the disease. Moreover, comparison of protein expression profiles found with that reported for SSc (Giusti *et al*, 2007b) shows large similarities. Altogether, these findings indicate the difficulty of the present knowledge to diagnose and monitor the sequel of SS development. This study is small and thus the results should be viewed as the first round and should be considered as a proof of concept.

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