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ORIGINAL ARTICLE

The minor salivary gland proteome in Sjögren's syndrome

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OBJECTIVE: To identify the global protein expression (the proteome) in the minor salivary glands from primary Sjögren's syndrome (pSS) patients and non-SS controls.

MATERIALS AND METHODS: Minor labial salivary glands were obtained from six pSS patients and from six age-matched non-SS controls, lysed in SDS buffer and pooled into two groups, respectively. The lysates were analysed by liquid chromatography electrospray ionization combined with tandem mass spectrometry. Also, the proteins were separated by two-dimensional polyacrylamide gel electrophoresis and protein spots were subjected to mass spectrometry.

RESULTS: Heat shock proteins, mucins, carbonic anhydrases, enolase, vimentin and cyclophilin B were among the proteins identified. The differences in the proteomes of minor salivary glands from pSS patients and non-SS controls were mainly related to ribosomal proteins, immunity and stress. Alpha-defensin-1 and calmodulin were among six proteins exclusively identified in pSS patients.

CONCLUSION: We have identified several minor salivary gland proteins that may have implications for clarifying the SS pathophysiology. This experiment adds to the knowledge of proteins produced in salivary glands in health and disease, and may form the basis of further studies on biomarkers of prognostic and diagnostic value.
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Introduction

Sjögren's syndrome (SS) is an autoimmune disease where lymphocytic infiltrates develop in the exocrine glands of patients, particularly the lacrimal and salivary glands (Jonsson *et al*, 2007). There is extensive local production of pro-inflammatory cytokines (Ek *et al*, 2006), chemokines attracting inflammatory mediators (Ogawa *et al*, 2002) and autoantibodies (anti-Ro/SSA and anti-La/SSB) (Salomonsson and Wahren-Herlenius, 2003). Lymphocytes are found to organize into ectopic lymphoid tissue in the salivary glands of SS patients, known as germinal centres (Salomonsson *et al*, 2003), mediating the inflammatory processes (Loetscher and Moser, 2002). The levels of immunoglobulins are elevated in serum (Wahren *et al*, 1994) and saliva (Sistig *et al*, 2002) of primary Sjögren's syndrome (pSS) patients.

In spite of extensive effort spent in tracing the underlying cause of SS, the aetiopathogenesis is still obscure. To elaborate on the pathophysiology of the disease, we and others have previously identified differentially expressed genes in the salivary glands of pSS compared to that in non-SS controls (Hjelmervik *et al*, 2005; Gottenberg *et al*, 2006). As the gene expression may not reflect the true biology of the glands, and the effector function in biological systems is at the protein level, it is imperative also to explore the salivary gland proteome, and to identify proteins of different quantities in the salivary glands of pSS and non-SS controls.

Liquid chromatography electrospray ionization combined with tandem mass spectrometry (LC-ESI-MS/MS) (Hu *et al*, 2005; Mann, 2007) and two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) combined with mass spectrometry (MS) (O'Farrell, 1975; Gorg *et al*, 2004) are well-established proteomic methods which allow for large-scale identification of hundreds of proteins in biological samples.

So far most efforts have focused on describing the proteome of whole saliva (Ghafouri *et al*, 2003; Vitorino *et al*, 2004a,b; Hardt *et al*, 2005; Hirtz *et al*, 2005; Hu *et al*, 2005; Guo *et al*, 2006; Walz *et al*, 2006) (reviewed by Amado *et al*, 2005). Saliva has the potential as a diagnostic fluid in several diseases, including pSS (Chiappin *et al*, 2007), and is obtained by non-invasive sampling. Potential pSS biomarkers, in particular

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proteins present exclusively in pSS, such as Ig kappa light chain, zinc-α2-glycoprotein and cyclophilin A, were identified in whole saliva (Giusti *et al*, 2007; Hu *et al*, 2007). Differentially expressed proteins have also been identified in parotid saliva from SS patients (Ryu *et al*, 2006).

For understanding what causes changes in saliva composition, and for a further insight into the SS pathology, mapping of the salivary gland proteome is imperative. Recently, Hu *et al* (2009) identified proteins by 2-D PAGE MS in parotid glands from pSS patients, pSS/MALT lymphoma and non-pSS control subjects, which supported the differential gene expression seen between the groups.

Our objective was to conduct a large-scale mapping of the proteome in salivary glands from pSS patients and control subjects using two complementary proteomic methods (LC-ESI-MS/MS and 2-D PAGE), and evaluate any differences observed between the samples. This preliminary investigation is a first step on the way to the mapping of the salivary gland proteome, which will serve as a basis for further efforts in discovering new biomarkers of diagnostic and prognostic value (for orally related diseases and SS in particular).

Materials and methods

Patients and controls

Minor salivary gland biopsies from the lower lip of pSS patients ($n = 6$), and from gender- and age-matched non-SS controls ($n = 6$) were obtained from the Department of Otolaryngology/Head and Neck surgery, Haukeland University Hospital. The biopsies were immediately frozen and stored in liquid nitrogen. The diagnosis of pSS was based on the American-European 2002 consensus criteria (Vitali *et al*, 2002). All pSS patients had subjective oral/eye sicca symptoms and a positive labial gland biopsy. Four of the patients had a focus score ≥2, of which two had positive serology, and two patients had a focus score of 1 and positive Ro and/or La serology. The pSS patients were all women with a median age of 53 years (range 40–80 years). The non-SS controls had subjective oral sicca complaints, but a negative biopsy and clinically no other signs or symptoms that justify a diagnosis of primary SS. The control group had a median age of 60 years (range 51–74 years).

Ethical approval was obtained from the Regional Committee for Medical Research Ethics (REK), Western Norway, no. 242.06.

Sample preparation

The minor salivary gland biopsies were washed in phosphate-buffered saline, blotted dry on a piece of tissue paper, submerged in liquid nitrogen and ground into fine powder by pestle and mortar. The powder was taken up in 500 μl 1% sodium dodecyl sulphate (SDS) PAGE buffer. The protein lysate was sonicated and vortexed, and centrifuged. The protein concentration was measured using the BCA Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA).

LC-ESI-MS/MS

The minor salivary gland lysates were combined to a pSS pool consisting of lysates from salivary glands of four of the pSS patients and a non-SS control pool with lysates from the six non-SS control salivary glands. Fifty micrograms of protein from each of the two pooled fractions was run separately on a NuPAGE® 12% Bis-Tris Pre-Cast Gel (Invitrogen, Carlsbad, CA, USA). The gel was stained with Coomassie blue (Bio-Rad Laboratories Inc.), and the lanes were cut into eleven gel pieces, as illustrated in Figure 1. The gel pieces were washed and dried in 50 μl 25 mM ammonium bicarbonate and 50% acetonitrile (ACN). The inherent proteins were then treated with 50 μl 10 mM DTT, followed by 50 μl 55 mM iodoacetamide and finally digested with trypsin (porcine; Promega, Madison, WI, USA), 180 ng in 30 μl digestion buffer (50 mM ammonium bicarbonate, 5 mM

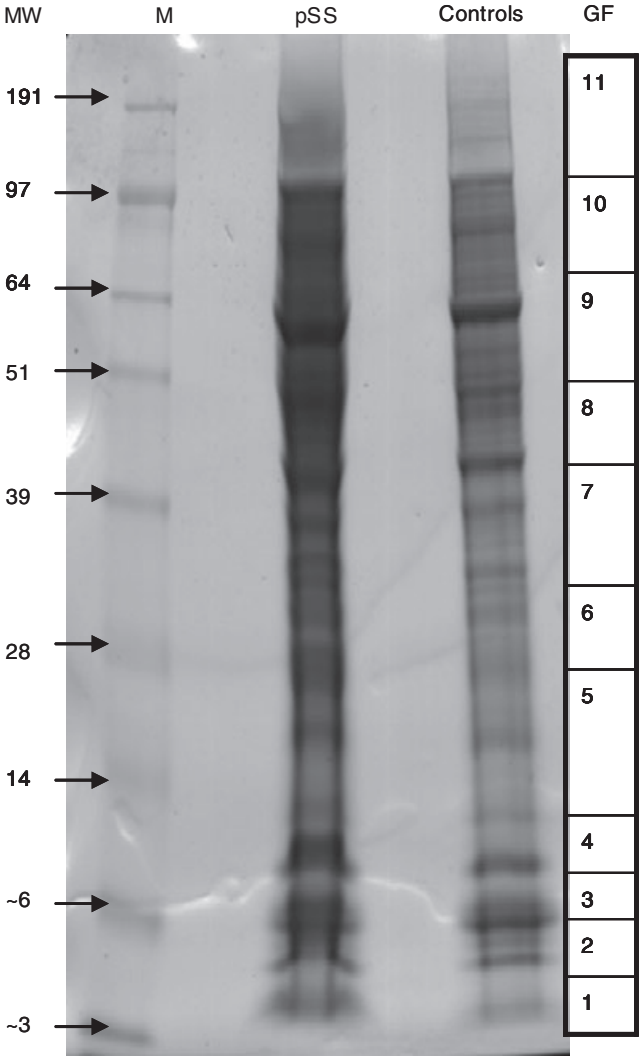

Figure 1 Proteins in total salivary gland lysate from primary Sjögren's syndrome patients (pSS) and non-SS controls were separated by electrophoresis on a NuPAGE® Novex 12% Bis-Tris gel in MOPS buffer. Molecular weight marker SeeBlue® Plus2 (M), with the molecular weight (kDa) indicated at the left side of the gel, and the eleven gel fractions for LC-ESI-MS/MS numbered at the right side

$CaCl_2$, 2% ACN), and incubated overnight at 37°C. The peptides were extracted from the gel pieces, and 20 μl from each of the 22 peptide fraction was applied to the nano-LC.

The LC-MS/MS of the peptides was performed using an Ultimate 3000 nano-LC (Dionex Corporation, Sunnyvale, CA, USA) coupled to an ESI-QTOF (Ultima Global, Waters, USA) operating in positive ion mode and equipped with fused silica capillaries (20 μm id) serving as spray emitters. The peptides were loaded via a nC18 Pepmap 100 enrichment column (300 μm $\times$ 5 mm; particle size 5 μm) to the nC18 Pepmap 100 analytical column (75 μm $\times$ 150 mm; 3 μm particle size) for nano-LC separation at a flow rate of 300 nl min^{-1}. The eluents used for the LC were (A) 2% ACN and 0.1% formic acid and (B) 90% ACN and 0.1% formic acid. A gradient was utilized from 5% to 50% B in 43 min and ramped to 95% B in 1 min. The column was washed for 15 min with 95% B and re-conditioned with 5% B for 15 min before the next run. The peptides were ionized at a voltage of 2950 V. Proteolytic peptide profiles were automatically acquired for the LC-MS runs, and MS to MS/MS switching by data-dependent analysis was applied for selecting and fragmenting the three peptides in the spectrum with the highest intensity. Collision energies for maximal fragmentation were automatically calculated by empirical parameters based on the charge and the *m*/*z*-value of the peptide.

Bioinformatics

MS/MS spectra were searched against the SwissProt database (http://www.expasy.org/sprot) version 51.3 consisting of protein sequences from *Homo sapiens* using Mascot Daemon as search engine (http://www.matrixscience.com) (Matrix Science, Ltd., London, UK). Variable and fixed modifications were set to oxidation (methionine) and carbamidomethyl (cysteine) respectively. One missing cleavage and a peptide tolerance level of 0.2 Da were allowed. The threshold peptide scores for identification of the proteins were calculated based on a level of significance of < 0.05.

The average peptide score was designated as protein score to show how well the protein was described by the peptides (Chepanoske *et al*, 2005). The results from the automated LC-ESI-MS/MS runs were validated by manual inspection of two MS/MS spectra of one protein from each peptide fraction.

The data are available in the PRIDE database (Martens *et al*, 2005) (http://www.ebi.ac.uk/pride) under accession numbers 7962 and 7963. The raw data from the LC-ESI-MS/MS experiment were made PRIDE compatible using the software PRIDE Converter v1.10.1 (http://www.code.google.com/p/pride-converter).

2-D PAGE

The samples were combined in two pools, where the minor salivary gland lysates from six pSS patients (100 μg from each sample) constituted one pool, and the minor salivary gland lysates from six non-SS controls (100 μg from each sample) constituted the other. The proteins in the pooled lysates were precipitated in chloroform/methanol, pelleted and air-dried. The pellet was dissolved in thiourea running buffer, consisting of 6 M Urea, 2 M Thiourea, 2% CHAPS and 2% IPG buffer, pH 3–10.

2-D PAGE was carried out as previously described elsewhere (Gorg *et al*, 2004), with some modifications. Strips with Immobilized pH Gradient (IPG) (GE Healthcare, Uppsala, Sweden) of 13 cm in length were rehydrated overnight in rehydration buffer (thiourea running buffer with 50 mM DTT). Samples were run in triplicate with approximately 100 μg of protein in thiourea running buffer with 50 mM DTT and 1% IPG buffer corresponding to the IPG strip. The first dimension isoelectric focusing parameters were: 150 V in 4 h, 300 V 4 h, 1000 V 6 h, 8000 V 3 h, 8000 V 4 h and 500 V 6 h.

The focused proteins in the IPG strips were incubated in equilibration buffer (6 M urea, 100 mM Tris–HCl, pH 6.8, 2% w/v SDS, 6% w/v Glycerol) containing 100 mM DTT for 15 min and subsequently in equilibration buffer containing 250 mM iodoacetamide for 15 min, and separated according to molecular weight on a 12.5% SDS polyacrylamide gel in a Hoefer SE600 Ruby electrophoresis chamber (GE Healthcare). The gels were immediately fixed in 10% methanol and 7% acetic acid for 1 h, stained with Sypro Ruby Protein Gel stain (Bio-Rad Inc.) overnight and scanned on the Typhoon 9400 (GE Healthcare).

The gel images were analysed using the Delta 2D software (Decodon, Greifswald, Germany). The two best gel replicates for each pH interval pH 4–7, pH 5.3–6.5 and pH 6–11 were used, and grouped according to pH interval and sample (pSS patient or control). The identified gel spots and the quantities (the mean percent volume, % vol) of the gel spots in the pSS patient gels were compared with the gels of the control group.

The gels were transferred to a black light source (Dark Reader; Clare Chemical Research, Dolores, CO, USA), and the spots selected by the image analysis were excised from the gels manually.

In gel tryptic digestion and peptide extraction

Tryptic digestion was done as described earlier with the following modifications; Trypsin (Promega), 90 ng in 30 μl digestion buffer (50 mM ammonium bicarbonate, 5 mM $CaCl_2$), was added to each gel piece for tryptic digest.

The peptides were adsorbed to an Empore 3M Extraction disk, C18 (Varian, Harbour City, CA, USA) and eluted with α-cyano-4-hydroxyl-cinnamic acid (CHCA) matrix in 60% ACN and 0.1% TFA, and spotted onto the MALDI target (Bruker Daltonics, Bremen, Germany). As a standard for the peptide mass fingerprint (PMF), 0.5 μl of Peptide Calibration Standard II (Bruker Daltonics) in one volume of CHCA matrix was used.

MALDI-TOF and PMF identification

The MALDI-target was inserted into an Ultraflex III TOF/TOF (Bruker Daltonics) and PMF was acquired by a 337-nm laser in reflector mode with accelerating voltage 20 kV, and delayed extraction parameter of 50 ns. Laser power was set to 10% above threshold

and spectra were obtained by summation of about 1000–1500 consecutive laser shots. The standard curve from the Peptide Mix II (in-house peptide standard) was used for assigning the mass to charge (m/z) values for the peaks in the spectrum, and the tryptic peptides were used for internal calibration of the PMF.

The obtained mass spectra were searched against the NCBI and SwissProt databases consisting of *Homo sapiens* protein sequences using MASCOT Daemon 2.1.0 as search engine (Matrix Science, Ltd.). The search parameters were mass tolerance 80 ppm, tryptic cleavage, maximum missing cleavages 1 and variable and fixed modifications were set to oxidation (methionine) and carbamidomethyl (cysteine) respectively. A protein with a significant Mowse score ($P < 0.05$) was regarded as a hit when the peptides in the PMF covered at least 20% of the complete protein sequence.

Western blot and immunohistochemistry

Proteins detected by LC-ESI-MS/MS and 2-D PAGE were also identified by Western blotting (Burnette, 1981) (antibodies listed in Table S1). Pooled lysates from patients and non-SS controls were run on 15% SDS-PAGE gels, blotted onto nitrocellulose membrane, and pre-incubated with primary antibodies (antibody concentrations are listed in Table S1). The horseradish peroxidase (HRP)-conjugated secondary antibodies, rabbit-anti-mouse-IgG (P207), swine-anti-rabbit (P217) and rabbit-anti-goat-Ig (P449), all from Dako (Dako, Carpinteria, CA, USA), were diluted in Tris-buffered saline 1:500, 1:3000 and 1:2000 respectively. The blots were developed by enzymatic conversion of DAB by HRP.

Immunohistochemistry was performed on sections from frozen salivary gland biopsies from pSS patients and non-SS controls. The technical aspects of immunohistochemistry were reviewed by Ramos-Vara (2005). Shortly, the salivary gland biopsies were fastened to bolts in Tissue Tek® (Sakura Finetek USA, Inc., Torrance, CA, USA) and stored at −80°C. Tissue sections of 4 μm were obtained using a cryostat at −20°C and adhered onto the charged surface of SuperFrost™ microscope slides (Erie Scientific Company, Portsmouth, NH, USA). The sections were fixed in acetone, incubated with anti-cyclophilin B antibody (Table S1) at a concentration of 2 μg ml^{-1} for 1–2 h and treated with H_2O_2 to abolish endogenous peroxidase activity. The antibody was detected using the DakoCytomation EnVision®+ Duallink system HRP (DAB+) kit, K4065 (Dako Cytomation, CA, USA), following the manufacturer's instructions.

Results

Large-scale delineation of the MSG proteome by LC-ESI-MS/MS

Salivary gland lysates were pooled for four of the pSS patients and for the six non-SS controls, and run separately in two lanes on a SDS-PAGE gel (Figure 1). The two gel lanes were cut in 11 corresponding sections and the inherent proteins were subjected to in-gel digestion by trypsin. The resulting peptides were extracted and a volume of 20 μl from each of the 22 fractions was loaded on the nano-LC enrichment column. We identified 431 proteins (non-redundant) in the 11 fractions from the pSS patients (Table S2), and 365 proteins (non-redundant) in the fractions from the non-SS controls (Table S3). Taken together, 522 proteins were identified (when combining the proteins identified in pSS and non-SS controls), out of which 158 in pSS only, 91 in non-SS controls only and 273 in common. A total of 251 proteins were identified by at least two peptides in either of the samples, and a subset of these having an average peptide score above 50 is presented in Table 1 (as well as Tables S2 and S3).

The proteins serum albumin and actin were highly abundant in the minor salivary glands of both pSS patients and non-SS controls. Among the identified proteins were mucin and carbonic anhydrases, and proteins related to inflammation, such as immunoglobulins, cyclophilins, heat shock proteins, macrophage migration inhibitory factor and β_2-microglobulin. Numerous 40S and 60S ribosomal proteins and cytokeratins were also identified.

For the proteins identified only in the pSS or the control sample (indicated in bold in Table 1) by LC-ESI-MS/MS, the spectra were manually inspected to ensure the absence of the peptides in the other sample. The following six proteins were exclusively identified in pSS as none of the peptides were detected in the control sample: neutrophil defensin 1 (Swiss Prot accession number: P59665), hypoxia up-regulated 1 (Q9Y4L1), calmodulin (P62158), up-regulated during skeletal muscle growth protein 5 (Q96IX5), all presented in Table 1, and proteasome activator complex subunit 1 (Q06323) and signal recognition particle 14 kDa protein (P37108) in Table S2.

Further identification of the minor salivary gland proteome by 2-D PAGE

The proteins from the pooled lysates were precipitated using the chloroform/methanol protocol, a method originally used for purifying lipid fractions from cell and tissue lysates (Blight and Dyer, 1959). This enabled us to obtain high resolution protein patterns in the gels and spots with defined boundaries, less streaking, and a high reproducibility of the spot pattern in the gels, which extensively increased the quality of the image analysis (Figure 2).

The majority of the proteome was located in the interval of pH 4–7, while the interval of pH 6–11 contained fewer proteins at lower quantities. This led us to increase the separation of the proteins by using the IPG strips with pH interval 5.3–6.5, enhancing the separability of the protein spots. Ninety-seven spots were picked for MS identification by MALDI-TOF and 63 distinct proteins were identified, of which 37 were unique proteins. The proteins identified are listed in Table 2, and Table S4, with the corresponding gels shown in Figure 2 and Figure S1. High abundance proteins, like serum albumin, actin and keratins, and immunoglobulins and cyclophilin B were identified. Additionally, 2-D PAGE enabled the identification of proteins not identified by LC-ESI-MS/MS, such as

Table 1 Proteins identified in minor salivary glands of patients and controls by LC-ESI-MS/MS

Sample[a]	*SP-ID*[b]	*Protein Name*[c]	*#P*[d]	*APS*[e]	*Gel section*[f]
P, C	P31946	14-3-3 protein beta/alpha (Protein kinase C inhibitor protein 1) (KCIP-1)	4	50	6
P, C	P63104	14-3-3 protein zeta/delta (Protein kinase C inhibitor protein 1)	5	76	6
P	**Q9Y4L1**	150 kDa oxygen-regulated protein precursor (Hypoxia up-regulated 1)	3	51	10
C	P62263	40S ribosomal protein S14	2	75	4
P, C	P62269	40S ribosomal protein S18 (Ke-3)	2	55	4, 5
P, C	P15880	40S ribosomal protein S2 (S4) (LLRep3 protein)	2	63	6, 7
P	**P60866**	40S ribosomal protein S20	2	51	3
P	P62851	40S ribosomal protein S25	2	70	4
P, C	P23396	40S ribosomal protein S3	4	62	6
P	**P46782**	40S ribosomal protein S5	4	53	5
P, C	P08865	40S ribosomal protein SA (p40) (34/67 kDa laminin receptor)	3	65	7
P, C	P10809	60 kDa heat shock protein, mitochondrial precursor (60 kDa chaperonin)	3	54	9, 10
P, C	P05388	60S acidic ribosomal protein P0 (L10E)	4	60	7
P, C	P62913	60S ribosomal protein L11 (CLL-associated antigen KW-12)	3	52	5
P	Q07020	60S ribosomal protein L18	3	57	5, 6
P	Q02543	60S ribosomal protein L18a	2	55	5
C	P46776	60S ribosomal protein L27a	2	57	5
P, C	P60709	Actin, cytoplasmic 1 (Beta-actin)	14	50	2, 4, 6–10
P	P59998	Actin-related protein 2/3 complex subunit 4 (20 kDa subunit)	2	54	5
P	**O15511**	Actin-related protein 2/3 complex subunit 5 (16 kDa subunit)	2	52	4
P, C	P14550	Alcohol dehydrogenase [NADP+] (Aldehyde reductase)	3	51	7
P, C	P12814	Alpha-actinin-1 (Alpha-actinin cytoskeletal isoform)	5	58	10
P, C	O43707	Alpha-actinin-4 (Non-muscle alpha-actinin 4) (F-actin cross-linking protein)	7	70	9–11
P, C	P06733	Alpha-enolase (2-phospho-D-glycerate hydro-lyase)	5	67	8
P, C	P04083	Annexin A1 (Annexin I) (Lipocortin I) (Calpactin II) (p35)	8	81	7, 8
P, C	P07355	Annexin A2 (Annexin II) (Lipocortin II) (Calpactin I heavy chain) (p36)	16	54	6–8, 10
P, C	P12429	Annexin A3 (Annexin III) (Lipocortin III) (Placental anticoagulant protein III)	7	63	7
P, C	P08758	Annexin A5 (Annexin V) (Lipocortin V) (Endonexin II)	9	63	6–9
P, C	Q8TD06	Anterior gradient protein 3 log precursor (AG-3 protein)	2	51	4
P, C	P02647	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I)	9	54	5, 6
P, C	P25705	ATP synthase subunit alpha, mitochondrial precursor	4	57	8, 9
P, C	P06576	ATP synthase subunit beta, mitochondrial precursor	9	60	8, 9
P	**O75964**	ATP synthase subunit g, mitochondrial (ATPase subunit g)	2	75	2
P, C	Q8N4F0	Bactericidal/permeability-increasing protein-like 1 precursor	2	59	8–11
P, C	P61769	Beta-2-microglobulin precursor	2	50	1, 2
P	**P62158**	Calmodulin (CaM)	2	58	4
P, C	P27824	Calnexin precursor (MHC class I antigen-binding protein p88)	2	57	9
P	P27797	Calreticulin precursor (CRP55) (Calregulin) (HACBP) (ERp60)	2	59	8
P, C	P00918	Carbonic anhydrase 2 (Carbonic anhydrase II)	6	57	6, 7
P	**P16152**	Carbonyl reductase [NADPH] 1 (Prostaglandin-E)	3	59	6
P	O00299	Chloride intracellular channel protein 1 (Nuclear chloride ion channel 27)	2	69	6
P, C	Q00610	Clathrin heavy chain 1 (CLH-17)	4	50	10, 11
P, C	P12109	Collagen alpha-1(VI) chain precursor	10	62	10, 11
P, C	P12111	Collagen alpha-3(VI) chain precursor	35	54	10, 11
P, C	P07585	Decorin precursor (Bone proteoglycan II) (PG-S2) (PG40)	3	58	9
P, C	P04843	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 67 kDa	2	66	9
P, C	P61803	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit DAD1	2	66	2
P, C	P68104	Elongation factor 1-alpha 1 (EF-1-alpha-1) (Elongation factor 1 A-1)	7	53	1, 2, 7–9
P, C	P13639	Elongation factor 2	3	60	10, 11
P, C	P14625	Endoplasmin precursor (Heat shock protein 90 kDa beta member 1)	16	55	9–11
P, C	P21333	Filamin-A (Alpha-filamin) (Filamin-1) (Endothelial actin-binding protein)	8	63	11
P, C	O75369	Filamin-B (Beta-filamin) (Actin-binding-like protein) (Thyroid autoantigen)	5	59	11
P	P30043	Flavin reductase (NADPH-dependent diaphorase)	2	57	5
P, C	P09382	Galectin-1 (Lectin galactoside-binding soluble 1) (Beta-galactoside-binding lectin L-14-I)	3	50	3
P, C	P06396	Gelsolin precursor (Actin-depolymerizing factor) (ADF) (Brevin)	4	59	9, 10
P	P09211	Glutathione S-transferase P (GST class-pi)	5	59	5
P, C	P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	8	59	5, 7, 8, 10
P	P63244	Guanine nucleotide-binding protein subunit beta 2-like 1	2	53	7
P, C	P08107	Heat shock 70 kDa protein 1 (HSP70.1)	5	55	2, 9, 11
C	**P08238**	Heat shock protein HSP 90-beta (HSP 84) (HSP 90)	2	56	10, 11
P, C	P04792	Heat-shock protein beta-1 (HspB1) (Heat shock 27 kDa protein) (HSP 27)	3	64	6
P, C	P68871	Haemoglobin subunit beta (Haemoglobin beta chain)	8	50	1–11
P	**P09651**	Heterogeneous nuclear ribonucleoprotein A1 (Helix-destabilizing protein)	3	61	7
P, C	P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	6	57	7
P, C	P10412	Histone H1.4 (Histone H1b)	4	51	7
P, C	P01876	Ig alpha-1 chain C region	6	66	9, 10
P, C	P01857	Ig gamma-1 chain C region	9	50	2–11
P	P01859	Ig gamma-2 chain C region	2	50	8–11
P, C	P01834	Ig kappa chain C region	5	55	3, 5, 6, 9
P	**P01620**	Ig kappa chain V-III region SIE -	2	83	6
P	P01842	Ig lambda chain C regions	4	51	6

Table 1 (Continued)

Sample[a]	*SP-ID*[b]	*Protein Name*[c]	*#P*[d]	*APS*[e]	*Gel section*[f]
P	P01871	Ig mu chain C region	7	57	9, 10
P	**P01591**	Immunoglobulin J chain -	3	61	5
P, C	O75874	Isocitrate dehydrogenase [NADP] cytoplasmic (Cytosolic NADP-isocitrate dehydrogenase)	4	67	8
C	**Q9NSB4**	Keratin type II cuticular Hb2 (Type II hair keratin Hb2) (Keratin-82)	2	51	6
C	**O43790**	Keratin type II cuticular Hb6 (Type II hair keratin Hb6) (Keratin-86)	4	56	6
C	**Q15323**	Keratin, type I cuticular Ha1 (Hair keratin, type I Ha1)	5	50	6
C	**Q14525**	Keratin, type I cuticular Ha3-II (Hair keratin, type I Ha3-II)	6	64	6
C	**Q92764**	Keratin, type I cuticular Ha5 (Hair keratin, type I Ha5)	3	53	6
P, C	P02533	Keratin, type I cytoskeletal 14 (Cytokeratin-14) (Keratin-14)	2	62	8
P, C	P05783	Keratin, type I cytoskeletal 18 (Cytokeratin-18) (Keratin-18)	7	52	7, 8
P, C	P08727	Keratin, type I cytoskeletal 19 (Cytokeratin-19) (Keratin-19)	10	60	7, 8,
P, C	P08729	Keratin, type II cytoskeletal 7 (Cytokeratin-7) (Keratin-7)	10	55	2, 6–10
P, C	P05787	Keratin, type II cytoskeletal 8 (Cytokeratin-8) (Keratin-8)	14	55	1, 2, 6–10
P, C	P02545	Lamin-A/C (70 kDa lamin) (NY-REN-32 antigen)	4	62	9
P, C	P00338	L-Lactate dehydrogenase A chain (LDH-A) (LDH muscle subunit)	3	81	7
P	P07195	L-Lactate dehydrogenase B chain (LDH-B) (LDH heart subunit)	3	56	7
P, C	Q7Z4W1	L-Xylulose reductase (Dicarbonyl/L-xylulose reductase) (Kidney)	2	65	6
P, C	P40925	Malate dehydrogenase, cytoplasmic (Cytosolic malate dehydrogenase)	3	53	7
P, C	P40926	Malate dehydrogenase, mitochondrial precursor	7	65	7
P, C	P15088	Mast cell carboxypeptidase A precursor (MC-CPA) (Carboxypeptidase A3)	4	53	2, 7
P, C	Q9HC84	Mucin-5B precursor (High molecular weight salivary mucin MG1)	16	59	3, 9–11
P, C	P60660	Myosin light polypeptide 6	4	55	3, 4
P, C	P35749	Myosin-11 (Myosin heavy chain, smooth muscle isoform)	9	53	11
P, C	P35579	Myosin-9 (Myosin heavy chain, non-muscle IIa)	12	58	10, 11
P	**O75489**	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	2	59	6
P	**P59665**	Neutrophil defensin 1 precursor (HNP-1) (Defensin, alpha 1)	2	60	1
P	Q9BZQ8	Niban protein	2	55	10
P, C	P23284	Peptidyl-prolyl cis-trans isomerase B precursor (PPIase) (Cyclophilin B)	8	50	2, 5
P, C	P30044	Peroxiredoxin-5, mitochondrial precursor (Peroxisomal antioxidant enzyme)	6	54	4
P, C	P30086	Phosphatidylethanolamine-binding protein 1 (Prostatic-binding protein)	6	66	2, 5
P	P15259	Phosphoglycerate mutase 2	3	55	6
P, C	Q15365	Poly(rC)-binding protein 1 (Alpha-CP1) (Nucleic acid-binding protein SUB2.3)	2	62	7
P, C	Q15084	Protein disulfide-isomerase A6 precursor (Protein disulfide isomerase P5)	7	64	8, 11
P, C	P07237	Protein disulfide-isomerase precursor	9	55	8–11
C	P60468	Protein transport protein Sec61 subunit beta	2	55	2
P, C	Q96DA0	Protein UNQ773/PRO1567 precursor	2	85	5, 6
P	**P62820**	Ras-related protein Rab-1A (YPT1-related protein)	3	55	5
C	P61019	Ras-related protein Rab-2A	2	77	5
P, C	Q00765	Receptor expression-enhancing protein 5 (TB2 protein)	2	64	4, 5
P	P52565	Rho GDP-dissociation inhibitor 1 (Rho GDI 1)	2	52	6
P	**Q13228**	Selenium-binding protein 1	2	59	8
P, C	P02768	Serum albumin precursor	39	53	1–11
P, C	P05023	Sodium/potassium-transporting ATPase alpha-1 chain precursor	6	57	10, 11
P	Q9Y490	Talin-1	2	62	11
P	**P10599**	Thioredoxin (ATL-derived factor) (Surface-associated sulphydryl protein)	2	64	2, 3
P	Q8NBS9	Thioredoxin domain-containing protein 5 precursor (p46)	3	53	8
P, C	P37837	Transaldolase	3	51	7
P, C	Q01995	Transgelin (Smooth muscle protein 22-alpha)	4	64	5
P, C	P51571	Translocon-associated protein subunit delta precursor (TRAP-delta)	2	51	3, 4
P, C	P60174	Triosephosphate isomerase (Triose-phosphate isomerase)	8	56	3, 5, 6
P, C	P68363	Tubulin alpha-ubiquitous chain (Alpha-tubulin ubiquitous)	6	63	7–9
C	P68371	Tubulin beta-2C chain (Tubulin beta-2 chain)	7	53	8
P, C	P22314	Ubiquitin-activating enzyme E1 (A1S9 protein)	4	58	10
C	Q13404	Ubiquitin-conjugating enzyme E2 variant 1 (UEV-1) (CROC-1)	2	50	4
P, C	P30085	UMP-CMP kinase (Cytidylate kinase) (Deoxycytidylate kinase)	2	52	5, 6
P	**Q96IX5**	Up-regulated during skeletal muscle growth protein 5 (HCV F-transactivated protein 2)	2	69	2
P, C	P08670	Vimentin	14	52	2, 7–11
P	P18206	Vinculin (Metavinculin)	3	51	10

Total protein lysates of minor salivary glands from patients with primary Sjögren's syndrome (P) and non-SS controls (C) were separated by SDS-PAGE, digested by trypsin and applied to LC-ESI-MS/MS analysis. The proteins were identified by searching with the MS/MS spectra in the Swiss-Prot database containing *Homo sapiens* protein sequences. Only proteins identified with two or more peptides and an average peptide score of 50 and above were included in the table. The remaining proteins are available in Tables S2 and S3.

[a]Sample pool where the protein was identified. C, controls; P, pSS.

[b]Swiss-Prot primary accession number, from the MASCOT search with the MS/MS spectra. Bold numbers indicate proteins identified in only one of the sample pools (either C or P), and no peptides of the protein in the opposite sample.

[c]From the MASCOT search. The proteins are sorted by the protein name.

[d]Number of peptides that the protein was identified with.

[e]Average peptide score. Average score of the peptides identifying a protein. For proteins identified in both sample pools (P, C), the APS from C was used.

[f]The fraction of the gel from which the protein was identified (see Figure 1). All gel fractions where the protein was identified are indicated.

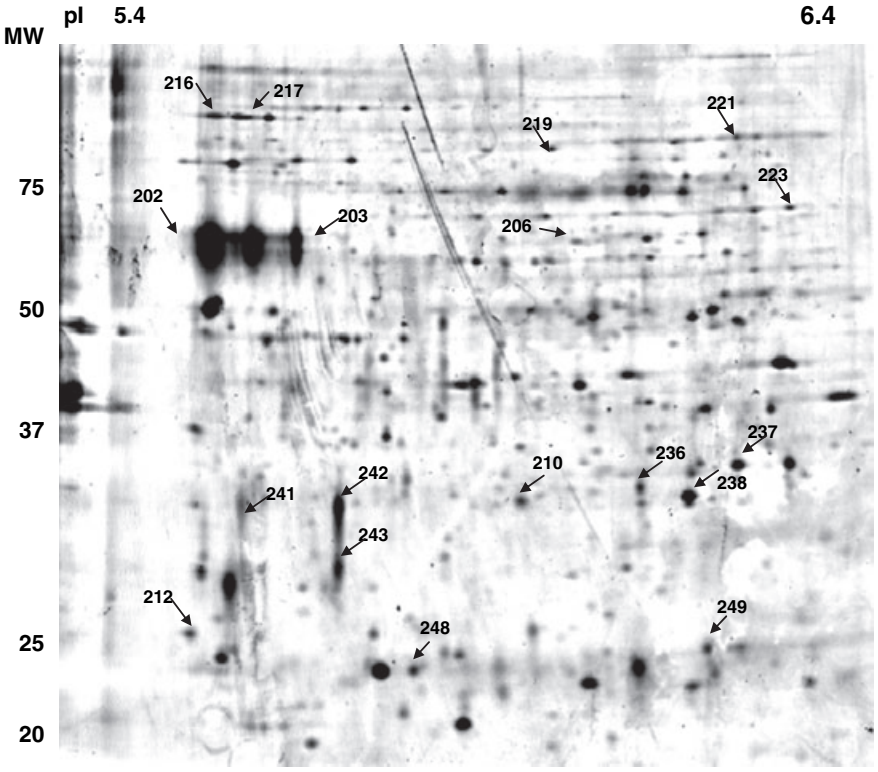

Figure 2 2-D PAGE of minor salivary gland lysate. The image shows the separation by 2-D PAGE of proteins in the pooled minor salivary gland lysate (100 μg) from non-SS controls in the pH interval pH 5.3–6.5. The gel was stained with SYPRO Ruby and is representative of two gel replicas. The Dual Color® Precision Plus Protein standard was used as molecular weight (MW) marker (kDa). The numbering corresponds to the spot number in Table 2

PARK7 (oncogene DJ-1), SLE autoantigen Ku70, enolase and interferon-induced protein IFIT-3.

The minor salivary gland protein lysates were run separately by 2-D PAGE, and differences in the spot pattern between the samples from pSS patients and non-SS controls were observed. Due to limited sample material, three replicates were run, of which only two gels from each group had reproducible spot pattern, and included in the analysis; therefore, the differences must be seen as semi-quantitative. To ensure homology between the spot patterns within the gel groups (pSS group and control group), spots with varying intensities (standard deviation $>50\%$) in the respective groups were removed from the analysis. After this filtration, about 500 spots per gel group were retained. In Figure S2, sections from the gels of the pH interval 5.3–6.5 are shown, and the protein spots 202, 203, 206, 210 and 242 which were identified as differentially expressed between pSS patients and non-SS controls are highlighted.

Overall, proteins related to matrix, metabolism and stress were found down-regulated in the pSS patients, while IgG molecules and serum albumin were up-regulated in pSS.

Verification of identified proteins by Western blot and immunohistochemistry

The presence of some of the identified proteins was verified by Western blot of the protein lysate and immunohistochemistry on minor salivary gland biopsy material from pSS patients and non-SS controls, which assured the presence of all the proteins selected for verification (Table S1 and Figure S3) in the minor salivary gland. Immunohistochemistry is illustrated in Figure S4, where the presence of Cyclophilin B showed localization to the acinar basal region, and not in the infiltrating cells or the ducts.

Discussion

To our knowledge, there has not been any comprehensive delineation of the salivary gland proteome, although there have been several studies of the saliva proteome (Ghafouri *et al*, 2003; Vitorino *et al*, 2004a,b; Amado *et al*, 2005; Hardt *et al*, 2005; Hu *et al*, 2005; Guo *et al*, 2006; Denny *et al*, 2008; Siqueira *et al*, 2008; Gonzalez-Begne *et al*, 2009), and for identification of possible SS biomarkers in saliva (Giusti *et al*, 2007; Hu *et al*, 2007). Our aim was to conduct a large-scale mapping of the minor salivary gland proteome, applying two complementary methods, namely, LC-ESI-MS/MS (Table 1 and Tables S2 and S3) and 2-D PAGE (Table 2 and Table S4).

Using this strategy, we identified hundreds of proteins from the minor salivary gland lysate. Due to the exploratory nature of this document and that the salivary gland proteome is expected to hold thousands of proteins, the proteins presented in this study represent only a small part of the complete minor salivary gland proteome. Serum albumin and ß-actin were abundant in the minor salivary gland samples of both pSS patients and non-SS controls, and the 40S and 60S ribosomal proteins were also prevalent. Furthermore, mucin 5B and protein kinase C inhibitor proteins (belonging to the group of 14–3–3 proteins) were identified. Previously, these proteins have all been reported in saliva (Hu *et al*,

Table 2 Proteins identified in minor salivary glands from pSS patients and non-SS controls by 2-D PAGE and MALDI-TOF MS

Spot[a]	*FC*[b]	*Protein ID*[c]	*Protein name*[d]	*SC (%)*[e]	*Score*[f]	*MW/pI*[g]	*MW/pI gel*[h]
102	0.65	gi|62414289	Vimentin	57	166	50, 5.2	50, 4.8
103		gi|31542947	Chaperonin (Hsp60)	37	121	61, 5.7	51, 5.3
104	0.58	gi|112180560	PDIA3	40	194	60, 6.6	50, 5.7
105	0.64	gi|89574029	Mitochondrial ATP synthase, H+ transporting F1 complex beta subunit	48	199	48, 5	48, 4.8
106		gi|32189394	ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit	45	180	56, 4.9	48, 4.9
107		gi|1710248	Protein disulfide isomerase	40	84	46, 5	48, 4.9
108		gi|4501885	Actin, beta	38	90	42, 5.3	45, 4.5
109		gi|15277503	Actin, beta	58	178	42, 5.3	42, 5.2
110		gi|15277503	Actin, beta	39	106	42, 5.3	42, 5.3
111	0.43	P08727	Keratin, type I cytoskeletal 19 (Cytokeratin-19)	53	316	44, 4.9	40, 4.9
112		P08727	Keratin, type I cytoskeletal 19 (Cytokeratin-19)	50	233	44, 4.9	39, 4.9
113	0.51	gi|90111766	Keratin 19	83	420	44, 4.9	38, 5.0
114		gi|90111766	Keratin 19	44	134	44, 4.9	35, 4.8
115		gi|999926	Annexin V	35	69	36, 4.8	32, 4.9
116		P31946	Protein kinase C inhibitor protein 1	30	84	28, 5	27, 4.6
117		P52565	Rho GDP-dissociation inhibitor 1 (Rho GDI 1)	33	87	23.2, 4.9	24, 4.9
119		gi|999926	Annexin V	47	129	36, 5.0	22, 5.5
121	0.41	gi|16507237	Hsp70	39	185	72.4, 4.9	71, 4.9
123		gi|55960304	Gelosin	26	86	86, 5.9	82, 5.8
124		P04792	HSP-beta1 (HSP27)	33	81	22.8, 6.0	23, 6.2
125		Q99497	PARK7(Oncogene DJ-1)	31	86	20, 6.3	22, 6.3
126		gi|34810822	Chain B-non-covalent complex, trypsin.	28	97	26, 8.23	18, 5.7
127		gi|34810822	Chain B, Non-Covalent Complex Between Alpha-1-Pi-Pittsburgh And S195a Trypsin	28	97	30, 9.3	15, 5.5
130		gi|55669910	Chain A, Crystal Structure of the Ga Module Complexed With Human Serum Albumin	20	132	71, 5.9	65, 5.8
131	2.3	gi|55669910	Complex A, Serum albumin	23	133	67, 5.6	65, 5.9
132	5.41	gi|11493459	PRO2619	20	97	59, 5.9	62, 5.9
133		gi|55669910	Chain A, Crystal Structure of the Ga Module Complexed With Human Serum Albumin	20	125	67, 5.5	65, 6.0
202	9.28	gi|55669910	Serum albumin, chain A	25.5	201	67, 5.5	67, 5.5
203	3.01	gi|119395750	Keratin 1	22	74	66.2, 8.6	67, 5.7
206	2.06	P12956	ATP-dependent DNA helicase 2 (Ku70)	21	68	70, 6.2	68, 6.1
208		gi|11935049	Keratin 1	34	96	66, 8.8	45, 5.5
210	3.15	gi|119582952	Annexin A1, isoform CRA_c	33.7	75.8	39, 6.7	30, 6.0
211	2.11	gi|4502101	Annexin I	49.7	202	39, 6.7	30, 6.3
212	2.57	P28070	Chain B, proteasome A ct reg (IAVOB)	40.7	70.6	20, 7.8	26, 5.5
213		gi|40354192	Keratin 10	23.6	86.6	60, 5	27, 5.8
218		gi|119593111	Family with sequence similarity 50, member A, isoform CRA_b (XAP5)	37.1	70	30, 5.8	80, 5.9
219		gi|40354192	Keratin 10	20.5	93	70, 5.0	80, 6.0
221		gi|109134330	PHD finger protein 20-like 1 isoform 1	28	95	75, 4.8	80, 6.3
223	0.37	gi|57014045	Lamin A/C transcript variant 1	34	190	75, 6.4	75, 6.4
236	0.18	gi|119615489	UDP-galactose 4-epimerase	31	118	35, 6.0	35, 6.1
237	0.16	gi|1633300	Aldehyde reductase	44	123	35, 6.4	37, 6.3
238	0.26	gi|4502101	Annexin I	43	148	39, 6.6	33, 6.2
242	0.22	gi|6650826	PRO2044	26	92	30, 7.7	35, 5.7
243	0.22	gi|6650826	PRO2044	23.7	65.7	30, 7.7	30, 5.7
246		gi|119626083	Albumin isoform CRA_t	21	126	60, 6.7	30, 5.5
248	0.28	gi|119626080	Albumin	31.7	96	26, 6.3	25, 5.8
249		gi|763431	Albumin	23	70	53, 5.6	25, 6.2
253		gi|119580906	HCG2010721	20	114	17, 5.6	15, 5.5
301	0.41	gi|114657944	Pyruvate kinase 3, isoform 2	29	112	58, 8.7	58, 9.0
302	1.9	gi|34782901	ATP5A1	32	147	49, 9.6	50, 8.6
303		gi|40354192	Keratin 10	20	77	59, 5.0	39, 6.2
305	1.7	gi|50368991	Ighg1	18.5	99	51.5, 9.3	50, 9.0
306	2.86	gi|50368991	Ighg1	24	113	52, 8.6	50, 9.2
307	4.21	gi|50368991	Ighg1	21	78	52, 9.4	49, 9.3
309	4.12	Q9Y6K8	KaD5 (Adenylate kinase isoenzyme 5)	29	56	22.3, 5.38	49, 9.8
319	0.64	gi|1310882	Cyclophilin	42.7	98	19.7, 9.7	15, 9.5
320	3.34	gi|3660145	Haemoglobin	63	125	15.7, 6.8	12, 7.0

Table 2 (Continued)

Spot[a]	*FC*[b]	*Protein ID*[c]	*Protein name*[d]	*SC (%)*[e]	*Score*[f]	*MW/pI*[g]	*MW/pI gel*[h]
322		gi|4503571	Enolase	25	112	48, 7.0	40, 7.2
324		gi|31645	Glyceraldehyde 3-phosphate dehydrogenase	27	71	36, 9.1	32, 9.0
325		gi|6648067	Malate dehydrogenase	38	99	36, 9.8	32, 9.5

Total protein lysates of minor salivary glands from patients with primary Sjögren's syndrome (pSS) and non-SS controls were separated by 2-D PAGE, digested by trypsin and applied to MALDI TOF analysis. The proteins were identified by searching with the MS spectra in the Swiss-Prot and NCBI databases using the Mascot Daemon search engine. Proteins with sequence coverage below 20% or with borderline Mowse score are available in the Table S3.

[a]Spot numbers are located on gels in the pH intervals pH 4–7 (≤ 199), pH 5.3–6.5 (200–299) and pH 6–11 (≥ 300), and can be viewed in Figure S1.

[b]Some of the spots were identified as differentially expressed by comparing gels run with SG protein lysate from pSS (pooled) and non-SS controls (pooled). Proteins with fold change values of >1 were up-regulated in pSS.

[c]The primary accession number retrieved from MASCOT search with the protein mass fingerprint (MS spectrum) in either NCBI or Swiss-Prot.

[d]Protein name retrieved from the MASCOT search.

[e]The protein sequence coverage.

[f]The Mowse score was based on a significance level of $P = 0.05$. The significant scores for PMF for NCBI and Swiss-Prot were 65 and 54 respectively.

[g]MW/pI: The theoretical molecular weight (MW) and isoelectric point (pI) of the protein are provided from the database search.

[h]The MW and pI from the spot-location on the gel (as seen in Figure S1).

2005, 2007; Walz *et al*, 2006), and recently in parotid gland exosomes (Gonzalez-Begne *et al*, 2009).

Several heat shock proteins were identified in the salivary glands by both methods. We identified heat shock proteins, HSP27, HSP60 and HSP90, which may be confined to the cellular entities of the tissue, where they function in the cytosol as chaperones in intracellular trafficking. The heat shock proteins are related to inflammation (Shovman *et al*, 2005; Chatterjee *et al*, 2007), and the level of autoantibodies against heat shock proteins was recently found to be lower in pSS patients than that in controls (Shovman *et al*, 2005), which indicate a role for heat shock proteins in the pathogenesis of pSS.

We identified several proteins in the minor salivary glands which have previously been identified in whole saliva, namely, cytokeratin 1, cytokeratin 10, carbonic anhydrase VI, cyclophilin B, enolase and HSPC059 (Walz *et al*, 2006). Carbonic anhydrase VI, enolase and several cytokeratins were recently identified in parotid gland exosomes (Gonzalez-Begne *et al*, 2009). Enolase is known to have a deteriorating effect on the oral cavity and has been proposed as an autoantigen in Hashimoto's encephalopathy (Yoneda *et al*, 2007) and cancer (He *et al*, 2007).

Cyclophilin B was present in the minor salivary glands from both pSS and non-SS controls. This was verified by immunohistochemistry of tissue sections from minor salivary gland biopsies. Cyclophilin B is known to function in the activation of interferon regulatory factor 3 transcription (Obata *et al*, 2005), and has been proposed to function as an inflammatory mediator in rheumatoid arthritis (De Ceuninck *et al*, 2003). Other reports have shown immunosuppressant properties for this protein, by interactions with cyclosporin A (Pap, 2005).

Differentially expressed proteins

To discover new biomarkers for diagnosis or targets for treatment in SS, one approach is to look for molecular signatures in the salivary glands and in saliva. This has been done previously on a gene expression level for the minor salivary glands (Hjelmervik *et al*, 2005; Gottenberg *et al*, 2006), and in proteomic studies on saliva (Ryu *et al*, 2006; Giusti *et al*, 2007; Hu *et al*, 2007) for identification of differentially expressed transcripts and proteins in SS patients *vs* controls. In a recent study by Hu *et al* (2009), genes and proteins differentially expressed in the parotid glands from pSS patients and non-pSS controls were identified.

The main objective of this work was to achieve a large-scale delineation of the minor salivary gland proteome in samples from both pSS patients and non-SS controls. In this process, we were also able to observe the differences between the samples. Several of the proteins were identified by two peptides or more in one sample, while absent in the other. By manual inspection of the LC-MS spectra of these proteins, we identified six proteins exclusively present in the pSS sample pool. These six proteins are important candidates to consider for further biomarker identification and validation in Sjögren's syndrome. Additionally, 2-D PAGE revealed differences in the protein profiles of pSS and non-SS controls. Although not statistically significant, our results are in line with previous reports on differences in the saliva proteome from pSS patients and non-SS controls.

One of the six proteins, alpha-defensin-1, exclusively identified in the pSS patients is known to have several roles in virus defence and is also shown to be up-regulating the type I IFN response genes (Falco *et al*, 2007), which was also a prominent finding in the gene expression profiles of the minor salivary glands from pSS patients (Hjelmervik *et al*, 2005; Gottenberg *et al*, 2006). This suggests an important role for alpha-defensin-1 in the pathology of SS, which was recently proposed as a salivary biomarker for oral inflammation in pSS patients (Peluso *et al*, 2007). Also calmodulin, only identified in the pSS patients, is a molecule with numerous biological roles ranging from regulation of

cellular processes to inflammation (Colomer and Means, 2007). Previously, elevated levels of calmodulin-binding proteins in the salivary glands from pSS patients have been reported (Deming *et al*, 2007). In particular, calmodulin is found to interact with Sjögren's syndrome autoantigen La/SSB (Castro *et al*, 1996), which can explain the elevated levels of calmodulin in the minor salivary glands of pSS patients.

Our results showed higher levels of immunoglobulins in the pSS salivary glands, where Ig kappa chain and Ig J chain were detected only in pSS by LC-ESI-MS/MS. The same pattern was seen by 2-D PAGE. The salivary glands of pSS patients is known as a place for formation of ectopic lymphoid tissue harbouring immunoglobulin producing plasma cells which undergo isotype switching and clonal expansion (Salomonsson *et al*, 2003; Bombardieri *et al*, 2007). Accordingly, hypergammaglobulinaemia is a hallmark of SS (Jonsson *et al*, 2007), which can explain the over representation of immunoglobulins in the minor salivary gland proteome from pSS patients. Previously, Ryu *et al* (2006) identified Ig kappa light chain to be up-regulated in saliva from pSS patients.

Other proteins involved in inflammatory processes, such as macrophage migratory inhibitory factor and stromal cell derived growth factor (IL25), were also identified in the pSS samples. Macrophage inhibitory factor was recently found to be increased in serum from pSS patients (Willeke *et al*, 2007), whereas IL25 has been found to induce Th2 responses in mice (Tamachi *et al*, 2006; Wang *et al*, 2007), and was recently found to regulate Th17 function in autoimmune diseases (Kleinschek *et al*, 2007).

Many of the 40S and 60S ribosomal proteins were only identified in the pSS minor salivary glands. These molecules are involved in the protein biosynthesis, and may indicate that the biosynthesis in pSS is increased compared with that in healthy control salivary gland, as corresponding to the increased levels of gene expression in pSS (Hjelmervik *et al*, 2005).

Molecules involved in metabolic processes, such as GAPDH, pyruvate kinase and glucosidase subunit B, were found to be down-regulated in pSS. The normal homeostasis in the salivary glands of SS is known to be deregulated, and the metabolic pathways are known to be reduced. Ramos-Casals *et al* (2007) found reduced metabolic function in serum of patients with pSS.

Annexins (I, II, III and V) were identified in both pSS patients and non-SS controls. Annexin I, which was found up-regulated in pSS compared with controls, is known to be involved in inflammation (D'Acquisto *et al*, 2008). Antibodies to these proteins have been identified in sera from patients with autoimmune diseases, such as RA, SLE and SS.

To our knowledge, this is the first time a mapping of the proteome of human minor salivary gland has been performed by LC-ESI-MS/MS and 2-D PAGE MS. Although this survey must be regarded as a preliminary study, it is a valuable contribution to further understanding of oral diseases in general and the SS pathology in particular, and provides a basis for identification of disease biomarkers.

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Author contributions

Hjelmervik contributed to the design of the study and the acquisition, analysis and interpretation of the data. He also drafted the manuscript. Jonsson obtained the biopsies, and contributed to the design of the study, analysis and interpretation of the data and critical revision of the manuscript. Bolstad provided the concept and contributed to the design, analysis and interpretation of the data and critical revision of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 2-D PAGE of minor salivary gland lysate.

Figure S2 Differentially expressed proteins in the salivary gland lysate.

Figure S3 Western blot verification of identified proteins.

Figure S4 Immunohistochemical staining of cyclophilin B.

Table S1 Antibodies used in Western blot verification of identified proteins.

Table S2 All 431 proteins identified by LC-ESI-MS/MS in minor salivary glands from Sjögren's syndrome patients.

Table S3 All 365 proteins identified by LC-ESI-MS/MS in minor salivary glands from non-SS controls.

Table S4 Proteins identified in minor salivary glands of pSS patients and non-SS controls by 2-D PAGE and MALDI-TOF MS.

Methods Detailed description of the methods used in this document.

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