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ORIGINAL ARTICLE

Comparative proteomic analysis of human oral fluids according to gender and age

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BACKGROUND: Recently, interest in finding disease bio-markers in human body fluids including oral fluids (OF), mainly saliva has increased. However, the physiologic differences in salivary proteins according to gender and age should be explored to establish a clinical diagnostic tool.

OBJECTIVE: To compare OF protein expression according to gender and age, using proteomic approaches.

MATERIALS AND METHODS: Oral fluids from 27 healthy volunteers (14 males, 13 females) was collected and divided into three age-groups. OF proteins were separated by means of 2D-SDS-PAGE. A total of 51 proteins in 37 protein spots were identified by ESI-MS/MS.

RESULTS: Gender differences revealed six proteins with significant higher expression in females, including β-2-microglobulin and transferrin. Age differences revealed decrease in expression of eight proteins with aging among males and seven proteins differentially expressed with aging among females including prolactin inducible protein, Ig-k light chain, transferrin, and calgranulin-B.

CONCLUSION: Proteomic analysis of OF revealed differences in protein expression according to gender and age and therefore can highlight future use of this technique for diagnostic purposes in health and in disease.

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Keywords: oral fluids; saliva proteins; gender; aging; 2-DE; ESI-MS/MS mass spectrometry

Introduction

Oral fluid (OF) is composed mainly of major and minor salivary gland secretions, gingival crevicular fluid, cell debris, microorganisms as well as bronchoalveolar and nasal secretions (Kaufman and Lamster, 2002). Every day more than 500 ml of saliva is secreted to the oral cavity, which is mostly reabsorbed through the gastrointestinal tract via swallowing (Pedersen *et al*, 2002). Saliva is mainly composed of water (99%); however, the smaller component but still crucial for many tasks is the bioactive molecules secreted in saliva (Baum, 1993). These molecules can be divided by their functional role such as assistance in food bolus formation, protecting against tooth demineralization, lubricating saliva, antiviral antibacterial and antifungal properties, and maintaining the normal equilibrium buffer state (Amerongen and Veerman, 2002). Therefore, saliva protein content is a complex mixture representing both the local and somewhat systemic condition of an individual.

Typically, body fluids, such as blood plasma, urine and cerebro-spinal fluid (CSF) are used for diagnostic purposes (Bergquist *et al*, 2002). The advantages of OF for diagnosis stem from its non-invasive, stress free, ample and simple collection (Kaufman and Lamster, 2002). These advantages open a new and a highly potential avenue towards seeking local and systemic diagnostic markers using proteomic composition of saliva. Previous studies have initiated the evaluation of the rich proteomic composition of saliva (Hardt *et al*, 2005; Streckfus and Dubinsky, 2007; Deutsch *et al*, 2008).

To date, more than 1000 different proteins have been identified in saliva (Hu *et al*, 2005; Guo *et al*, 2006; Denny *et al*, 2008), A recent study by Srivastava *et al* reported gender differences in the level of gene expression related to salivary gland proteins (Srivastava *et al*, 2008). Nevertheless, to our knowledge, protein composition differences using proteomic analysis related to gender and age have not been addressed. It is known that many major differences in the physiology of men and women result from the activity of gonadal steroid hormones. Animal research as well as human studies has revealed gender differences in both composition and flow rate of saliva (Lukacs and Largaespada, 2006).

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Age-related changes have been reported in healthy individuals regarding salivary composition and gland morphology (Nagler, 2004). Animal studies have revealed reduction in protein synthesis with aging (Vissink *et al*, 1996). Histologic analyzes have demonstrated that with aging the parenchyma of the salivary glands is gradually replaced by adipose and fibrovascular tissue, and the acini become atrophied with reduction in its volume (Azevedo *et al*, 2005; Moreira *et al*, 2006). Although some functional studies among healthy individuals indicate that aging itself does not necessarily lead to diminished glandular capacity to produce saliva, and although salivary glands still function in old age (Tylenda *et al*, 1988; Fischer and Ship, 1999), it is expected that saliva composition has changed.

We hypothesize that OF may possess a unique pattern when assessed according to gender and age. The aim of this study was to compare the proteomic composition of OF in healthy males and females according to gender and age.

Materials and methods

Subjects and oral fluids

The OF accumulation protocol was approved by the Ethical Committee of Hadassah Medical Center, Jerusalem, Israel.

Inclusion criteria consisted of healthy adults (>18 years old) not taking any medication including oral contraceptives, with no complaint of oral or ocular dryness and no oral mucosal lesions. Patients with removable dentures or smokers were excluded as well. All of the volunteers were requested not to eat, drink or brush and wash their teeth for 1 h prior to the trial. Measurements were performed between 8 A.M. and 12 A.M. by a single examiner (Y.F.).

Using the spitting method (Aframian *et al*, 2006), unstimulated OF from 27 individuals was collected into a precalibrated tube for both sets of experiments, i.e., age and gender. Exclusion criteria included no history of autoimmune disease, no history of irradiation therapy or malignancies, and no symptoms of xerostomia. Individuals were asked to wash their mouth for 30 s with water to remove cell debris and blood ingredients from potential gingival bleeding. After another 10 min of rest, OF collection was obtained sitting in an upright position and in a quiet room. During the measurement, volunteers were asked not to speak nor leave the room.

Samples were kept on ice and immediately 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,1976) (Bio-Rad, Hercules, CA, USA). The supernatants were frozen at −70°C and lyophilized overnight. Sediments were dissolved in 7 M urea, 2 M thiourea, and 4% CHAPS and stored at −20°C until analyzed.

To discover gender differences in salivary proteome, we compared six adult males' and six adult females' aged-matched samples (25.5 ± 2.4, 24.7 ± 1.9) by 2-DE analysis followed by MS identification. To discover age differences, samples of three age groups (adult, middle-aged and aged) were analyzed. The adult age group was composed of eight subjects randomly picked out of the 12 subjects used for the gender differences. To avoid gender interference, we compared male's age groups and female's age groups separately (Table 1).

2-DE

We used 2-DE methodology to detect protein expression differences between the experimental groups as a basic and powerful method for protein spot quantification followed by MS identification (for review on different saliva proteins analysis methods see Bigler *et al*, 2009). The criteria for choosing the protein spots for MS identification were based on two parameters: (i) spots intended to confirm our data in comparison to previous 2-DE maps (Ghafouri *et al*, 2003; Yao *et al*, 2003; Vitorino *et al*, 2004; Hu *et al*,2005). For this reason, we picked protein spots with a wide range of *Mr* and *pI*, while excluding multiple protein isoforms; (ii) Spots which display different intensities regarding gender and age.

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 DryStrip gels (Amersham Biosciences, Uppsala, Sweden) as previously described (Fleissig *et al*, 2009). Re-hydration was carried out 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 for 16 h, with a constant voltage of 50 V at 20°C using a Protean IEF Cell (Bio-Rad). The voltage was then gradually increased to 10 000 V at 20°C, and samples were focused for an additional 8 h. To prepare the gel strips for separation in the second dimension, the strips were soaked twice for 15 min in 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 traces of bromophenol blue and loaded onto hinged spacer plates (20 × 20.5 cm; Bio-Rad) using 9–16.5% SDS polyacrylamide gradient gel. Electrophoresis was conducted simultaneously for the samples on the same running

Table 1 Male and female subjects' characteristic in age comparisons. Age ranges of the groups were characterized according to age limits found at: http://www.ncbi.nlm.nih.gov/sites/entrez

		Male		*Female*	
	No. (M/F)	*Age (Range)*	*Mean ± s.d.*	*Age (Range)*	*Mean ± s.d.*
Adult	4,4	22–26	24.2 ± 1.7	23–25	24.2 ± 0.9
Middle-Age	4,4	42–46	43.7 ± 1.7	43–49	45.5 ± 2.6
Aged	4,3	78–88	82.7 ± 4.2	66–74	70 ± 4

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 gels were stained with ammoniacal silver nitrate using the Dodeca Stainer Shaker (Bio-Rad).

Image and statistical analysis
Gels were scanned using a computer GS-800 calibrated densitometer (Bio-Rad), and protein spots were detected and their intensities quantified using PDQuest software V 6.2.0 (Bio-Rad). To semi-quantify spot intensities and to minimize staining, variation between gels normalization was performed using the human serum albumin (Swiss-Prot accession number P02768) housekeeping protein. Expression levels of spots were determined by the relative spot volume of proteins compared with the average volume of two spots in the gel and expressed as a relative volume intensity. *T*-test was used to evaluate protein expression levels according to gender and age. The proteins differently expressed with statistical differences were selected and identified.

Mass spectrometry identification and database searching
For mass spectrometric identification, representative 2D sodium dodecyl sulfate–polyacrylamide gel electrophoresis of a young male containing 520 μg proteins 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 of interest were sterilely excised from the representative gel, washed with 100 mM ammonium bicarbonate, pH 8.0, and were reduced with 45 mM dithiothreitol for 30 min at 60°C for in-gel digestion. Alkylation was performed by adding 100 mM iodoacetamide in the dark for 30 min and the solution was discarded. Gel pieces were shrunk by 50% acetonitrile, 50% ammonium bicarbonate 100 mM, dried with 100% acetonitrile and followed by a Speed-Vac. Gel pieces were rehydrated with a solution of sequencing grade trypsin (Promega, Madison, WI, USA), 10 μg/ml in 25 mM ammonium bicarbonate, and the digestion was carried out for 16 h to 20 h at 37°C. Peptides were extracted from the gel three times by the addition of 2 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 $ZipTip_{C18}$ (Milliopore, Bedford, MA, USA) cleanup step that was performed according to the manufacturer's protocol. The ZipTip eluent was injected into a Qtof2 (Micromass, Manchester, England) equipped with a nanospray capillary and analyzed by ESI-MS/MS. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mapping is most commonly used for high through-put protein identification applications, however it has limited accuracy. Thus, to obtain accurate protein identification, we used the electrospray tandem mass spectrometric analysis to identify the proteins.

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. 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 NCBI data bank (http://www.ncbi.nlm.nih.gov) using the Mascot package (Matrix Science, London, England). In addition, data were also confirmed according to the Swiss-Prot protein database (http://www.expasy.ch).

The experimental fragment ion masses were matched with theoretical peptide fragment ion masses within the window of experimental mass measurement accuracy of $\pm$ 0.3 Da.

Results

Proteomic mapping of human oral fluids
Approximately, 300 protein spots were detected on silver stained 2DE gels (Figure 1 depicts the healthy young male map). Thirty-seven spots of interest were picked, trypsinated, and identified by ESI-MS/MS. Out of these spots, 51 proteins were identified. The number of matched peptides, the sequence coverage, and the ion scores were used to assess the credibility of the database search results (Table 2).

Proteomic differences according to gender
Out of the 300 protein spots detected on the silver stained 2DE gels, we were able to detect six protein

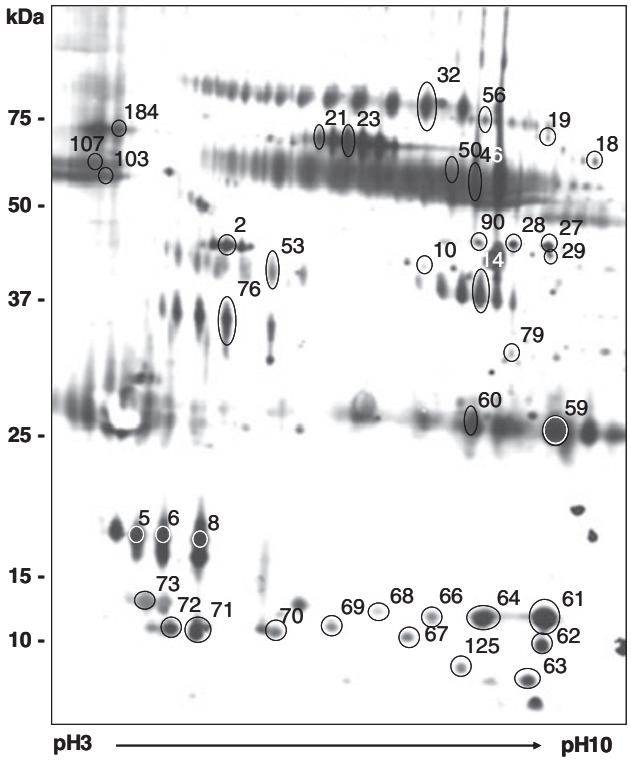

Figure 1 Depicts a two-dimensional electrophoresis (2-DE) gel map of a young male's OF. Protein spots identified by MS are circled and numbered

Table 2 Protein spots identified; Theoretical (Theor.) p*I*, *MW*. Ion scores are calculated as −10*Log (*P*), where *P* is the probability of the observed match to be a random event. Individual ion scores > 88 indicate identity or extensive homology. Protein scores are derived from the ion scores as a non-probabilistic basis for ranking protein hits

Spot	*Protein identification*	*Accession no.*	*Theor. Mw (Da)*	*Theor. pI*	*Matched peptides*	*Sequence coverage (%)*	*Ion score**
2	β-actin	P60709	41664	5.15	14	40	678
5	Prolactin-induced protein	P12273	16562	8.26	10	56	490
6	Prolactin-induced protein	P12273	16562	8.26	8	49	311
8	Prolactin-induced protein	P12273	16562	8.26	10	56	386
10–1	Leukocyte elastase inhibitor	P30740	42715	5.90	13	38	702
10–2	α-s1-casein	P47710	24513	4.98	3	19	176
14	Carbonic anhydrase VI	P23280	35345	6.51	11	27	527
18	Transketolase	P29401	67751	7.90	9	22	363
19–1	Transferrin	P02787	77030	6.97	14	29	446
19–2	Complement component 3 precursor	P01024	187046	6.02	5	4	96
21	Serum albumin precursor	P02768	69180	5.91	22	30	513
23	Serum albumin precursor	P02768	69180	5.91	20	31	811
27	âenolase	P06733	47139	7.01	15	46	1201
28	âenolase	P06733	47139	7.01	9	35	798
29	Phosphogluconate dehydrogenase	P52209	53219	6.80	20	43	623
32	Polymeric immunoglobulin receptor	P01833	83232	5.58	12	20	638
46	Salivary α-amylase	P04745	55857	6.21	17	51	1201
50–1	Salivary α-amylase	P04745	57714	6.32	5	43	948
50–2	IgA (Ig -1 chain C region)	P01876	50581	9.34	2	8	116
53–1	Zn-α-2-glycoprotein	P25311	34714	5.71	13	46	520
53–2	Serum albumin precursor	P02768	52048	5.69	2	5	88
56–1	Transferrin	P02787	77030	6.97	17	22	414
56–2	Polymeric immunoglobulin receptor	P01833	83232	5.58	10	15	218
59	Ig κ light chain	P01834	23466	4.99	15	55	831
60	Ig κ light chain	P01834	23466	4.99	11	55	633
61	Cystatin-SN precursor	P01037	16351	6.82	14	60	496
62–1	Cystatin B	P04080	11167	7.90	6	55	256
62–2 Ig -1	Chain C region	P01857	49174	8.80	4	13	215
63	Calgranulin A	P05109	10945	9.19	11	47	308
64	Cystatin-SN precursor	P01037	16351	6.82	9	47	430
66–1	Fatty acid binding protein 5 epidermal	Q01469	15155	6.60	8	40	279
66–2	Cystatin-SN precursor	P01037	16351	6.82	2	24	116
67–1	Keratin, type II cytoskeletal 1	P04264	65978	8.16	6	11	341
67–2	Cystatin D	P28325	16070	6.70	5	44	291
67–3	Keratin 10, type I, epidermal	P13645	57213	5.01	3	7	153
67–4	Cytokeratin 9	P35527	62092	5.19	3	8	110
68	Cystatin-SN precursor	P01037	16351	6.82	9	56	385
69	Calgranulin-B	P06702	13102	5.71	13	56	579
70	Calgranulin-B	P06702	13102	5.71	9	55	348
71–1	Prolactin-induced protein	P12273	16562	8.26	11	63	469
71–2	Calgranulin-B	P06702	13102	5.71	2	24	100
72	Prolactin-induced protein	P12273	16562	8.26	11	63	482
73	Cystatin SA-III	P01036	14181	4.74	12	71	519
76–1	Parotid secretory protein (SPLUNC2)	Q96DR5	26995	5.35	10	42	577
76–2	Similar to common salivary protein 1	Q96DA0	18867	5.38	2	18	150
79	Carbonic anhydrase VI	P23280	35345	6.51	9	33	539
103	Salivary α-amylase	P04745	57731	6.47	20	55	1279
107	Salivary α-amylase	P04745	57714	6.32	15	47	782
125–1 β-2	Microglobulin	P61769	12791	5.77	6	40	144
125–2	Calgranulin A	P05109	10931	9.19	2	34	103
184–1	Serum albumin	P02768	69349	6.13	13	26	705
184–2	Similar to common salivary protein 1	Q96DA0	18867	5.38	4	32	241

spots with significantly higher (> 2-fold) expressions in the female group compared with the male group (Figure 2).

The highest difference was obtained in β-2 microglobulin and/or calgranulin A (accession no. P61769; P05109) demonstrating a 5-fold increase. The transferrin and/or polymeric immunoglobulin receptor (accession no. P02787; P01833) showed a 4-fold increase. Calgranulin A and/or Ig κ-light chain (accession no. P05109; P01834) demonstrated a 3-fold increase followed by the leukocyte elastase inhibitor and/or α-s1-casein (accession no. P30740; P47710), which increased by 2-fold. Another unidentified spot showed a 2-fold increase.

Proteomic differences according to age

In the male group out of the 300 protein spots detected on the silver stained 2DE gels, we found eight proteins that demonstrated decreased expression with aging: β-actin (accession no. P60709), prolactin-induced protein (accession no. P12273), parotid secretory protein

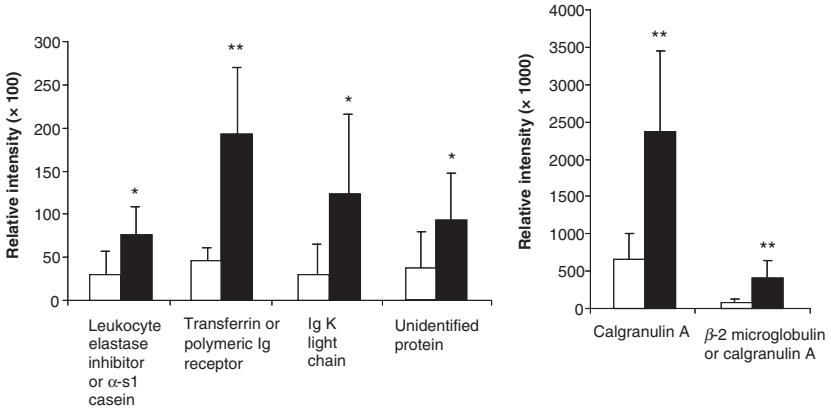

Figure 2 Relative expression of protein spots according to gender; Mean volume intensity in male group (□) and female group (■). Significant differences between males and females according to *T*-test (*$P \leq 0.05$, **$P \leq 0.01$)

(accession no. Q96DR5), calgranulin-B (accession no. P06702), cystatin-SN (accession no. P01037), phosphogluconate dehydrogenase (accession no. P52209), carbonic anhydrase (accession no. P23280), and Ig-κ light chain (accession no. P01834) (Figure 3).

In the female group, we found that the expression of prolactin inducible protein and cystatin-SN decreased with aging. Expression of Ig-κ light chain, β-actin and transferrin (accession no. P02787), or C3 and carbonic anhydrase were found to increase with aging. Expression of α-enolase (accession no. P06733) was found to increase in middle-aged individuals and decrease in the aged female group (Figure 4).

Discussion

OF is a mixed solution originating from different sources, mainly secretion of the major salivary glands. Saliva is an ultra-filtrated fluid from the blood stream. To date, more than 2000 proteins have been identified in saliva (~1.7/1.1 fold more than in CSF/Urine, respectively) using proteomics (Beeley and Khoo, 1999; Amerongen and Veerman, 2002; Ghafouri *et al*, 2003; Yao *et al*, 2003; Huang, 2004; Vitorino *et al*, 2004; Hu *et al*, 2005; Guo *et al*, 2006; Denny *et al*, 2008; Li *et al*, 2009). In this study, we used a small number of males and females to demonstrate changes in their OF protein components according to age and gender. Nevertheless, it was possible to detect significant changes, some of which were confirmatory to the findings shown recently (Ambatipudi *et al*, 2009) for aged changed parotid female saliva components, i.e., changes in carbonic anhydrase and immune related proteins. Using small groups allows the detection of major changes, and therefore further investigation using large group of human subjects with strict health general and oral health status recording will allow revealing significant changes in other moderately changed protein components. Furthermore in some cases when proteomic technology yields ambiguous results for instance when proteins migrate together, in 2-DE, other confirmatory technologies should be utilized. All of these are needed for the future OF use as an accepted diagnostic tool.

Regarding proteomic differences according to gender, we found six protein spots with significantly higher expressions in the female group compared with the male group, from these the changes in β-2 microglobulin and transferrin are of special interest. β-2 microglobulin is the β-chain of the major histocompatibility complex class I molecules found on the surface of white blood

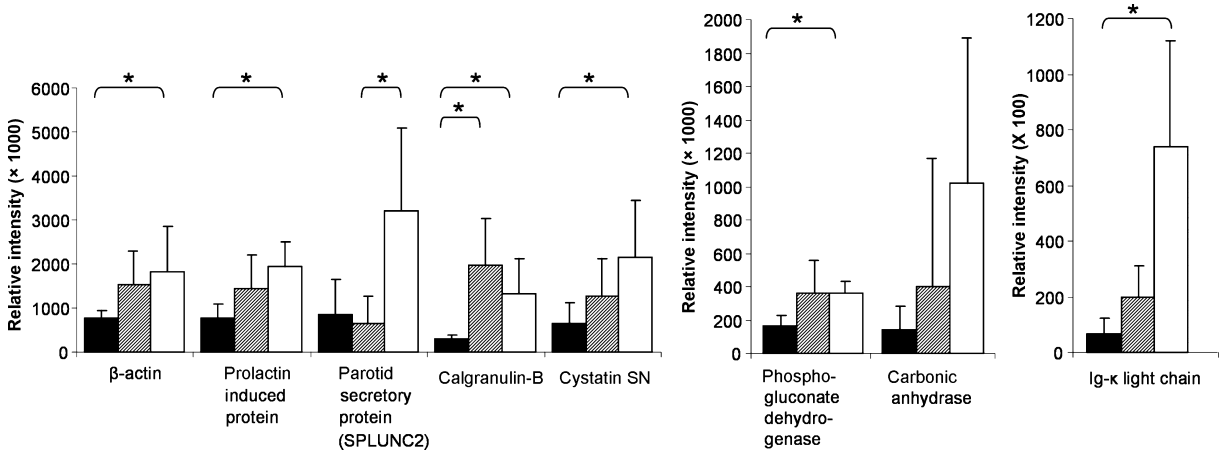

Figure 3 Relative expression of protein spots according to age among males; Mean volume intensity in adult (□), middle-aged (▨), and aged (■). Significant differences between age groups according to *T*-test (*$P \leq 0.05$)

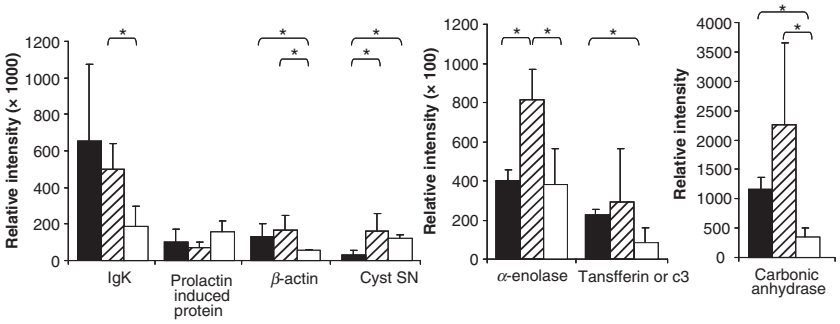

Figure 4 Relative expression of protein spots by age of females; Mean volume intensity in adults (□), middle-aged (▨), and aged (■). Significant differences between age groups according to *T*-test (*$P \leq 0.05$)

cells. This significantly higher expression of β-2 microglobulin in women's OF is interesting as high levels of this protein were found in patients suffering from two autoimmune disorders: Sjögrens' syndrome and systemic lupus erythematosus. These autoimmune disorders are found predominantly in females and 9-fold higher in the case of the Sjögren's syndrome (Castro *et al*, 2003). Furthermore, as the incidence of non-Hodgkin's lymphoma in Sjögrens' syndrome patients is up to 44 times more than in the healthy population (Ehrenfeld *et al*, 2001), it will be intriguing to further investigate the role of β-2 microglobulin in these patient's OF.

At present, there is no single clinical or laboratory marker that correlates absolutely with Sjögrens' syndrome. Moreover, a previous study found two patterns of salivary protein expression with no direct relation to the clinical, serological, or histologic severity of Sjögrens' syndrome patients (Fleissig *et al*, 2009). Therefore, refinement of Sjögrens' syndrome by aging and/or gender may attribute to more specificity in diagnosing and monitoring the progress of this exocrinopathy.

Transferrin is an iron binding transport protein. Higher levels of serum transferrin suggest an anemia type iron deficiency. It is well established that during the menstrual cycle many women show higher levels of transferrin (Williams, 1995). Moreover, hormonal effects such as estrogen induce higher transferrin levels in plasma (McKnight *et al*, 1980). In our study, significantly higher levels of transferrin were found in OF. All women were in their fertility age and were not taking any contraceptive drugs. However, we did not record whether the collection of the subjects' OF was taken during their menstrual period. Transferrin is considered a routine test in cases of suspected anemia. Consequently, further work is needed to analyze the validity of OF transferrin protein levels as an indicative test for anemia in relation to gender.

Regarding our second aim, i.e., to detect proteomic differences according to age, we found 8 proteins with decreased expression among aged males and two among females, three proteins with increased expression among aged females and one protein with variable profile expression increased in middle-aged individuals and thereafter decreased in the aged group. From these, the changes in prolactin inducible protein, Ig-κ light chain, transferrin, and calgranulin-B are of special interest.

Prolactin inducible protein was ~2.5-fold decreased in aged males compared with the adult male group and is a protein that functions in human reproductive and immunologic systems. Its exact function is still not known; however, various possible functions have been proposed such as inhibition of bacterial growth, immune regulation, and formation of enamel pellicle by binding to hydroxyapetite. Prolactin inducible protein is expressed in several exocrine tissues such as the lacrimal, salivary, and sweat glands. Its expression is up regulated by prolactin and androgens and down regulated by estrogen (Hassan *et al*, 2009). Estrogen levels decrease with aging, and this might be the reason for the 2.5-fold decrease from the adult to the aged females.

Immunoglobulin-κ light chain is probably a part of sIgA. It was found that IgA concentrations increase with aging (Nagler and Hershkovich, 2005). This is consistent with our results which revealed a 5-fold increase in aged females compared with adults. However, the male group showed opposite results with an 18-fold decrease in the aged compared with the adult. This inconsistency in aging variations between males and females is well established in literature as some studies demonstrate impairment of the immune system in old age (Vissink *et al*, 1996), and others (Vissink *et al*, 1997; Tanida *et al*, 2001) report salivary sIgA reduction with aging. This issue should be further explored, as well as the possible effects of hormones on salivary sIgA levels.

Transferrin as discussed earlier in this study is a serum protein that indicates body iron stores. Reduction of transferrin in saliva was reported with aging (Nagler, 2004). Interestingly, we found that transferrin increased with aging among females by more than 3.5-fold (aged compared with adult). This indicates iron deficiency among the middle-aged and the aged. It is possible that middle-aged females still had menstrual period, however we did not record this. As for the aged females, it is possible that they had insufficient iron due to their daily diet.

Calgranulin-B decreased by 3.5-fold in aged males compared with adult males. It is an inflammatory-associated protein expressed in neutrophils and other cells. The serum level of calgranulin-B increases in chronic inflammatory conditions. As immunologic defense systems are reduced in healthy elders (Vissink *et al*, 1996), perhaps this could be the reason for the decreased expression of calgranulin-B in saliva.

Conclusion

In this preliminary study, we compared the protein composition of OF in healthy volunteers by gender and age. In the course of this study, we found gender-related differences in six proteins, all over-expressed in the female group. Further work should be conducted to clarify these observations and to explore the beneficial role of these proteins in healthy and unhealthy subjects.

Considering aging effects, we found expression alterations in a few proteins, which function in saliva immune responses, cell metabolism, buffer capacity, and normal physiologic processes. These proteins are believed to change their expression as part of the aging process. Although the biologic basis of aging is unknown, it is possible that gene alterations, damage accumulation of biologic processes, interference with metabolism, and free radicals are involved. Further research is needed to shed light on the relevant mechanisms, especially as the number of elderly people has been dramatically increasing over the past few decades, and geriatric pathology is becoming more important in clinical practice.

The ability to track changes in expression levels by proteomics highlights the feasibility of using OF as a sensitive and convenient method for monitoring individual systemic protein bio-markers.

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