Published in final edited form as:

J Proteome Res. 2009 November ; 8(11): 5093–5102. doi:10.1021/pr900478h.

Quantitative Analysis of Age Specific Variation in the Abundance of Human Female Parotid Salivary Proteins

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Summary

Human saliva is a protein-rich, easily accessible source of potential local and systemic biomarkers to monitor changes that occur under pathological conditions; however little is known about the changes in abundance associated with normal aging. In this study, we performed a comprehensive proteomic profiling of pooled saliva collected from the parotid glands of healthy female subjects, divided into two age groups 1 and 2 (20–30 and 55–65 years old, respectively). Hydrophobic charge interaction chromatography was used to separate high from low abundant proteins prior to characterization of the parotid saliva using multidimensional protein identification technology (MudPIT). Collectively, 532 proteins were identified in the two age groups. Of these proteins, 266 were identified exclusively in one age group, while 266 proteins were common to both groups. The majority of the proteins identified in the two age groups belonged to the defense and immune response category. Of note, several defense related proteins (e.g. lysozyme, lactoferrin and histatin-1) were significantly more abundant in group 2 as determined by G-test. Selected representative mass spectrometric findings were validated by western blot analysis. Our study reports the first quantitative analysis of differentially regulated proteins in ductal saliva collected from young and older female subjects. This study supports the use of high-throughput proteomics as a robust discovery tool. Such results provide a foundation for future studies to identify specific salivary proteins which may be linked to age-related diseases specific to women.

Keywords

Parotid Saliva; Hydrophobic Charge Interaction Chromatography; Mass Spectrometry

Introduction

Saliva is an exocrine secretion, the majority of which is produced by the parotid and submandibular glands ¹. Saliva contains a large and diverse set of proteins which perform multiple functions such as lubrication, taste and digestion, maintenance of mucosal integrity, pH buffering, tooth mineralization and maintenance of general oral health by interacting with a complex collection of oral microbiota. Changes in the expression of these proteins alter the

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Author contributions. K.S.A. and F.K.H. designed experiments; K.S.A. and B.L. performed experiments; K.S.A. and B.L. analyzed data; and K.S.A., F. K. H., B.L., J.E.M., and J.R.Y. wrote the paper.

Supplementary data. Table 1, 2, 3 are provided.

normal functional properties of saliva; such changes would be expected to reflect systemic or local illness. Alterations in salivary protein composition can be monitored using diagnostics techniques and compared with other clinical parameters (serum, urine, and biopsy) ^{2–4}. Consequently, saliva's easy access and non-invasive collection procedures make it an ideal diagnostic fluid to monitor oral and systemic health.

A recent study found significant age-related differences in gene expression in the human female parotid gland, based on transcriptional analysis ⁵. However, only limited, and often conflicting, age-related differences have been noted at the proteomic level. In fact, some studies have reported no significant changes with age in the composition ⁶, salivary flow, and buffering capacity ⁷ of saliva. In contrast, mucins decreased ^{8, 9}, while transforming growth factor- α , IgG and IgA increased with age ^{10, 11} in whole saliva. No such age-associated changes in parotid saliva were observed for total protein content, amylase, lactoferrin, secretory IgA and proline-rich proteins ^{12–14}. However, other studies reported an increase in total protein, secretory IgA and lactoferrin ^{13, 15} and a reduction in amylase activity with age in parotid saliva ¹³.

Women are more frequently affected by autoimmune diseases which affect salivary gland function; e.g. Sjögrens syndrome and systemic lupus erythematosus ^{16, 17}. Recent comprehensive studies using mass spectrometry have reported nearly two thousand proteins in saliva ^{18, 19}, suggesting that it has the complexity to serve as a diagnostic fluid. High-throughput mass spectrometry has proven to be a successful strategy to detect changes in protein expression that are linked to specific disease conditions in other organ systems (e.g. lung, prostate and ovary) ^{20–22}, and consequently, these differentially expressed proteins may serve as sensitive biomarkers for better diagnosis and treatment. Not only are the frequency, onset and progression of many diseases age- and gender-dependent, but there is a natural age-dependent expression of marker proteins. Consequently, the first step towards the development of diagnostic tests requires an understanding of the protein expression profiles of healthy individuals at different ages.

The aim of the present study was to identify both the differentially and commonly expressed salivary proteins in healthy young (20–30 years old) and older (55–65) females. Parotid saliva was fractionated using hydrophobic charge interaction chromatography (HCIC) to reduce complexity prior to characterization by mass spectrometry (MS). Quantitative analysis of protein expression was performed by high-throughput MS analysis using multidimensional protein identification technology (MudPIT) ²³. G-test spectral counting quantitation was performed in a pair-wise comparison between the two groups. Fifty percent of the 532 proteins detected in saliva were expressed in both age groups. These results demonstrate that protein expression in the saliva of females is age dependent, thus it is critical to take into consideration these normal differences in protein expression when searching for clinically relevant, disease specific biomarkers.

Materials and Methods

Saliva Collection

The protocol for the collection of human parotid saliva by informed consent was approved by the University of Rochester Institutional Review Board. Fourteen adult, non-smoking females were screened for good general and oral health with normal salivary function and not taking any medications known to affect salivary gland secretion, including hormone replacement therapy. Subjects were divided into two age groups of seven individuals each ranging from 20–30 yrs (group 1), and 55–65 yrs (group 2). Group 1 subjects were pre-menopausal (normal monthly menstrual cycles) while all individuals in group 2 were post-menopausal (no menstrual cycles). Parotid saliva was collected on ice as ductal secretions under stimulation (0.4% citric

acid period of 30–120 min into sterile tubes) using a Lashley cup-like device²⁴ between 9:00 AM and 2:00 PM. Immediately after collection, 1/20 volume of protease cocktail inhibitor (0.1M Tris-HCl, pH7.4, 0.1M epsilon amino caproic acid, 0.05M sodium EDTA, 25 mg/L of pepstatin A, 0.025M benzamidine-HCl, 0.25 mg/L of leupeptin and 0.5M phenylmethylsulfonyl fluoride) was added to the saliva to prevent proteolytic degradation. Collected saliva was stored at -80°C until analysis.

Large Scale Protein Fractionation of Parotid Saliva by Hydrophobic Charge Interaction Chromatography (HCIC)

To normalize the differences between subjects and to reduce individual variation, parotid saliva collected from each of the seven subjects within an age group were pooled (approximately 70ml). Pooled parotid saliva was subjected to HCIC performed on a Biologic DuoFlow Maximizer purification system (Bio-Rad, Hercules, CA, USA) using a 2 ml column with 4-mercaptoethylpyridine (MEP Hypercel) as ligand, which at high concentration adsorbs via hydrophobic interactions without use of high concentration of lyotropic salts²⁵. Proteins binding to the ligand was carried out at neutral conditions followed by desorption at low pH, thus inducing an ionic charge causing repulsion between the ionizable ligand and bound protein²⁶. Prior to loading onto the column, pooled parotid saliva from each group was clarified by passing it through a 0.22 μm filter to remove particulate material. The MEP Hypercel column was first equilibrated with 10 ml of 50mM Tris HCl pH 8.1 followed by saliva loading. After the sample was completely loaded onto the column, the weakly bound proteins were washed with 10 ml of 50 mM Tris HCl pH 8.1. The chromatographic run was monitored at 214 nm for protein detection and flow-through was collected as 5 ml fractions for subsequent analysis as shown in Fig. 1A, Fig. 2A and Fig. 3A. For elution of proteins, column flow was reversed and a 20 ml gradient of 50 mM Tris HCl pH 8.1 to 50 mM sodium acetate pH 4 (100: 0) followed by 50 mM Tris HCl pH 8.1: 50 mM sodium acetate pH 4 (0: 100) in 20 min was applied to the column, followed by 24 ml of 50 mM sodium acetate pH 4 over 25 min. The eluted fractions (1ml each) were collected into tubes containing 100 μl of 50 mM sodium phosphate pH 9.1. Finally, 40 ml of 50 mM Tris HCl pH 8.1 was applied to re-equilibrate the column for subsequent fractionations. All buffers and saliva samples were filtered, degassed and loaded at a linear velocity of 1ml/min.

Analysis of Fractions on a Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Pooling and Dialysis

After chromatographic separation, an aliquot (10 μl) from each saliva fraction was removed and analyzed on a 4–20% Tris-glycine SDS-PAGE gradient gel (Invitrogen, Carlsbad, CA, USA). Gels were run at 200 V, until the bromophenol blue front had completely run off the bottom of the gel. Subsequently, the gels were stained with Simply Blue stain (Invitrogen) and images captured (Alpha Innotech, San Leandro, CA, USA).

Fractions collected from the flow through (pool A; amylase and histatins depleted), wash (pool B), and a pH elution (pool C) were pooled separately for dialysis using a 3.5 kDa molecular weight cut-off membrane. Pools A, B & C were dialyzed against four changes of water or 40 mM ammonium bicarbonate at 4°C . After dialysis all pools were lyophilized prior to characterization by mass spectrometry.

Protein Processing and Trypsin Digestion

The lyophilized fractions were dissolved in 100 mM Tris-HCl buffer pH 8.5. The protein concentration was determined using the BCA (bicinchoninic acid) protein assay kit (Bio-Rad) as per manufacturer's instructions. The samples were then reduced by adding 20 mM final concentration of Dithiothreitol (DTT) and cysteines were alkylated by adding 20 mM final concentration of iodoacetamide (IAA). Subsequently, the samples were digested overnight at

37°C in a final concentration of 2M urea with 100 mM Tris-HCl, pH 8.5 containing trypsin at an enzyme/substrate ratio of 1:75 (Promega, Madison, WI, USA). The reaction was stopped by addition of 90% formic acid to a final concentration of 4%. Digested samples were stored at -80 °C until mass spectrometry analysis.

Multidimensional Protein Identification technology (MudPIT)

Fractions (pools A and C) isolated from each of the two age groups were treated identically for identification by MudPIT, while pool B was separated by reverse phase before identification. Samples were run in triplicate, except for pool B which was run in duplicate. One hundred microgram samples of digested protein were pressure-loaded for fractions from the different age groups (pools A1 & A2 and C1 & C3) onto a fused silica capillary column packed with 3 cm of 5- μ m Partisphere strong cation exchanger (SCX, Whatman, Clifton, NJ, USA) and 3 cm of 5- μ m Aqua C₁₈ material (RP, Phenomenex, Ventura, CA, USA), with a 2 μ m filtered union (UpChurch Scientific, Oak Harbor, WA, USA) attached to the SCX end. The column was washed with buffer containing 95% water, 5% acetonitrile and 0.1% formic acid. After desalting, a 100- μ m i.d. capillary with a 5- μ m pulled tip packed with 10 cm 5- μ m Aqua C₁₈ material was attached to the filter union, and the entire split-column was placed inline with an Agilent 1100 quaternary HPLC (Agilent, Palo Alto, CA, USA) and analyzed using a modified 12-step (for samples A1, A2, and A3) or 6-step (for samples C1, C2, and C3) separation described previously²³. Three buffer solutions were used: 5% acetonitrile/0.1% formic acid (buffer A); 80% acetonitrile/0.1% formic acid (buffer B); and, 500 mM ammonium acetate/5% acetonitrile/0.1% formic acid (buffer C). The first step consisted of a 75 min gradient from 0 to 100% buffer B. Steps 2–6 had the following profile: 3 min of 100% buffer A; 7 min of X% buffer C; a 5 min gradient from 0 to 10% buffer B; and, a 65 min gradient from 10 to 45% buffer B. For 12-step separation, the 5 min buffer C percentages (X) were 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%, respectively, for the 2–12 step analysis. For 6-step separation, the 5 min buffer C percentages (X) were 10, 30, 50, 75 and 100%, respectively, for the 2–6 step analysis.

Fractions B1 & B2 had lower amounts of protein and were therefore separated by reverse phase (C₁₈) column only before MS. Fifty microgram samples of protein were pressure loaded for fractions of B (B1 & B2) onto a 10 cm 5- μ m Aqua C₁₈ column and the entire split-column was placed inline with an Agilent 1100 quaternary HPLC. Digests were eluted using a linear gradient of H₂O:CH₃CN (98:5, 0.1 % formic acid) to H₂O:CH₃CN (50:50, 0.1 % formic acid) at 300 nL/min over 120 min.

As peptides were eluted from either type of the microcapillary column, they were electrosprayed directly into an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) with the application of a distal 2.5 kV spray voltage. A cycle of one full-scan mass spectrum (400–1400 *m/z*) followed by six data-dependent tandem mass (MS/MS) spectra at a 35% normalized collision energy was repeated continuously throughout each step of the multidimensional and single phase separation. Application of mass spectrometer scan functions and HPLC solvent gradients was controlled by the Xcalibur data system (Thermo Fisher Scientific).

Interpretation of Tandem Mass Spectra Data Sets and functional classification of proteins

The ProLuCID algorithm was used for MS/MS database search²⁷. Tandem mass spectra were searched against EBI International Protein Index (IPI) protein database version 3.48 (September 2008) concatenated to a decoy database in which the sequence for each entry in the original database was reversed²⁸. ProLuCID results were assembled and filtered using the DTASelect (version 2.0) program^{29, 30}. DTASelect 2.0 uses a linear discriminant analysis to dynamically set XCorr and DeltaCN thresholds for the entire dataset to achieve a user-specified

false positive rate (5% at the protein level in this analysis). Proteins identified by the same peptide sets were clustered together by DTASelect 2.0. The DTASelect 2.0 program assembles identified peptides into proteins and protein groups by using a parsimony principle in which the minimum set of proteins accounts for all the observed peptides. A protein was deemed acceptable as a confident match based on the minimum number of two unique peptide matches from a combined pool of MS/MS from triplicate analyses for pools A & C and duplicate analyses for pool B. Protein annotation was obtained using UniProtKB/Swiss-Prot databases (sourced from www.expasy.org).

Assessing Differential Protein Abundance Using G-test Spectral Counting

The spectral counts for each protein were normalized according to total spectral counts for all the proteins in the samples. Let t_1 be the total spectral counts for all the proteins in sample 1, t_2 be the total spectral counts for all the proteins in sample 2, n_1 be the spectral count for the protein in sample 1, n_2 be the spectral count for the protein in sample 2, then

$$f_1 = n_1 \frac{t_2}{t_1}, \quad f_2 = n_2,$$

where f_1, f_2 are the normalized spectral counts for the protein in sample 1 and sample 2, respectively. The normalized spectral counts were then further corrected using Yates's correction³¹,

$$f^*_1 = f_1 + \text{sign}(f_1 - \hat{f}_1) \times 0.5, \quad f^*_2 = f_2 + \text{sign}(f_2 - \hat{f}_2) \times 0.5,$$

where, $\hat{f}_1, \hat{f}_2$ are expected spectral counts for the protein in sample 1 and sample 2, respectively. We assume that the protein is equally expressed (null hypothesis), thus $\hat{f}_1 = \hat{f}_2 = (f_1 + f_2)/2$. The function $\text{sign}(a-b)$ returns 1 if $a > b$, 0 if $a = b$, -1 if $a < b$. Essentially, for the Yates's correction, we subtracted 0.5 from each observed spectral count value that was greater than the expected, and we added 0.5 to each observed spectral count value that was less than the expected.

The G-value was calculated as described previously³²:

$$G = 2f^*_1 \ln \left(\frac{f^*_1}{\hat{f}_1} \right) + 2f^*_2 \ln \left(\frac{f^*_2}{\hat{f}_2} \right),$$

The calculated G-value was then used to assess whether the protein was differentially expressed according to the chi-square (χ^2) distribution table with one degree of freedom. Proteins with G-value larger than 6.635 are differentially expressed with $P < 0.01$.

Western Blot Analysis

Five μg of protein from each group was separated in NuPAGE® Novex 4–12% Bis-Tris polyacrylamide gradient gels (Invitrogen). Protein was transferred onto nitrocellulose membranes (Pall Corporation, Pensacola, FL, USA) for 1 h using 1X NuPAGE transfer buffer. Membranes were blocked for 1h with 5% non-fat dry milk with 0.05% Tween 20 (TBST) at room temperature. Subsequently blots were probed with antibodies against carbonic anhydrase VI (R&D Systems, Minneapolis, MN, USA) or amylase (Abcam, Cambridge, MA, USA) and developed using ECL western blotting detection reagents (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK). The amylase antibody from Abcam was generated against

full-length human pancreatic amylase which is almost identical to human salivary gland amylase.

Results

Separation of Abundant Proteins by Hydrophobic Charge Interaction Chromatography (HCIC)

Parotid ductal saliva was collected from 14 healthy female subjects and divided into two different age groups for analysis (group 1, 20–30 yrs; and group 2, 55–65 yrs). To improve the detection of low abundant proteins, the most abundant proteins (amylases and histatins) were depleted from parotid saliva by hydrophobic charge interaction chromatography (HCIC). SDS-PAGE analysis demonstrated that abundant proteins were separated from less abundant proteins for Groups 1 and 2 (Fig. 1A and Fig. 2A, respectively). Lane ST (starting material) contains ~10 ug of protein from unfractionated pooled saliva prior to addition to the HCIC column. Note the presence of intense protein bands representing amylases and histatins (as indicated by the arrows) with the exception of fractions 3–16 (pool A). The majority of the amylases and histatins were successfully separated and subsequently eluted in fractions 18–53 (pools B and C) in both age groups. In addition to amylase and histatins, a few faintly stained proteins were also observed in the elution fractions.

Protein Identification and Classification

A total of 338 and 460 proteins were identified with high confidence from 20–30 and 55–65 yrs age groups, respectively. Protein matches were deemed confident with the detection of at least two unique peptides from a combined pool of MS/MS from multiple analyses performed for each pool. As a group, 528 non-redundant proteins were identified in the two age groups. Of these proteins, 266 (50%) were detected exclusively in a single age group, while the remaining proteins were common to both age groups (Fig. 3).

Each identified protein was categorized into a single classification using a scheme based on the broad functional properties of proteins, sourced from www.expasy.org, although some proteins have multiple functions. Of the 338 proteins identified from group 1 (20–30 yrs), and displayed in Fig. 4A, more than 80% of the proteins belonged to one of four functional classifications including defense (11.6%), immune component (34%), enzyme (29.2%) and regulatory (9.4%). The remaining proteins (<20%) were present in 7 other functional categories, e.g. transport, secretory and development. The identified proteins for group 1 are listed in Supplementary Table 1.

The largest number of proteins (460) was identified in group 2 (55–65 yrs; Supplementary Table 2). Similar to age group 1, the vast majority (>80%) of the proteins detected in age group 2 belonged to the immune function (40%), enzyme (24.8%), regulatory (10.2%) and defense (7.8%) categories (Fig. 4B). The remaining proteins were divided into 8 functional groups that corresponded to those found in group 1.

Quantitative Analysis of Differentially Expressed Proteins

Spectral counting was used to quantitate changes in protein abundance between the two age groups identified above. A curated protein list from the two age groups was compiled, and a G-test on the spectral counts for each protein was performed. All proteins listed in Supplementary Table 3, including 832 redundant and non-redundant proteins with a calculated $G > 6.635$ ($P < 0.01$), were used for the comparative analysis. In a comparison between group 1 versus 2 (20–30 vs 55–65 yrs), we documented 293 differentially expressed proteins. The comparison resulted in the identification of 88 proteins that were more abundant in group 1, while the remaining 205 proteins were expressed at a lower level in group 1. Note that Group

2 expressed significantly more proteins (such as IGHG1, IGKC and Ig kappa chain) in higher abundance than Group 1.

A pair-wise comparison of differentially expressed proteins that belonged to defense, enzymes, immune component and regulatory activities was also performed (Fig. 5A), while a fewer number of differentially abundant proteins were identified for other functional groups as shown in Fig. 5B.

Validation of Proteomic Results by Western Blot Analyses

To confirm and quantify the differentially abundant proteins in the two age groups, western blot analysis was performed on two selected proteins. Validation of the MS and G-test results was performed with proteins expressed differentially (carbonic anhydrase VI) or constitutively (amylases) in the two age groups (Fig. 6). The three variants of carbonic anhydrase VI (CA VI) were similarly expressed in group 1 (18,055 spectral counts), while its abundance was more than 50% less in group 2 (8,600 counts) (Fig. 6A). In contrast to CA VI, the total spectral counts for the five different variants of amylase detected in the saliva of the two age groups (group 1 = 52,629 and, group 2 = 61,468 counts, respectively) and abundance (Fig. 6B) remained relatively constant between the 2 age groups. The relative abundance profiles, as determined by western blot analysis, were thus similar to the quantitative spectral counts data obtained for these 2 proteins with age.

Discussion

Blood has historically been used as the primary routine diagnostic medium for analyzing cellular, molecular and chemical changes associated with pathological conditions. However, the exploration of saliva as a diagnostic fluid has recently gained momentum due to its easy accessibility and non-invasive collection procedures that require little equipment and training. Notably, systemic diseases may cause changes in salivary constituents; e.g. increased levels of proteins such as cytokines and interleukins in Sjögrens syndrome (SS)³³. One important consideration not always adequately controlled for in past studies is that many diseases are age dependent^{34–36}. Consequently, the present study was designed to identify the differentially and commonly expressed salivary proteins in healthy young and older females as an initial step towards the development of saliva-based clinical tests.

This is the first comprehensive, high-throughput proteomic study to identify and quantify the global changes in the abundance of salivary proteins associated with normal aging in healthy females. Tandem liquid chromatography-mass spectrometry methods have a significant advantage for resolving proteins and peptides over 2-dimensional gel electrophoresis methods^{37–39}. In the present study HCIC was used for the separation of proteins (see Materials and Methods). Consequently medium and low copy proteins were unmasked from abundant proteins like amylases and histatins, which were identified using an LC-ESI-MS/MS approach that significantly enhanced its identification using Multidimensional Protein Identification technology (MudPIT)²³. The relative abundance of the identified salivary proteins was quantitated by G-test spectral counting. To increase confidence of our protein identifications, protein identities were deemed acceptable only when two unique peptides were detected for each protein. The overall false positive rate at the protein level was below 5%.

More than 900 proteins were recently identified in parotid saliva by mass spectrometry¹⁸, 349 of which overlapped with the 532 proteins detected in the present study. Although many proteins were common to both age groups, a surprisingly large number of proteins (50%) were uniquely expressed in an individual age group. The highest number of proteins was identified in the older age group (Fig. 3). It is interesting to note that the majority of the 183 proteins that did not overlap with the previously catalogued parotid saliva proteome¹⁸, were identified in

the older age group, study subjects that were under represented in this earlier study. Consequently, our results significantly expand the number proteins in the human parotid saliva proteome and emphasize that it is important to consider normal, age-related changes in protein abundance when using saliva as a diagnostic fluid.

One of the primary functions of saliva is to protect the upper gastrointestinal tract from infection. Accordingly, the largest number of proteins identified in both age groups was those associated with immunological protection (~45%; Defense and Immune component categories). These immune-related proteins could be further subdivided into proinflammatory, anti-inflammatory and antimicrobial proteins. Common to the saliva in two age groups, the immunoglobulin-related proteins, members of the immunoglobulin superfamily (IgSF) such as Ig kappa chain V-III region VH and kappa light chain, have a role in immune protection⁴⁰. Salivary IgA was the predominant external secretory immunoglobulin isotype. Secretory IgA acts as the first line of defense against pathogens which colonize or invade mucosal surfaces⁴¹. The high valency of SIgA enables it to agglutinate bacteria, neutralize viruses, enzymes and toxins^{42, 43}. SIgA antibodies generated in response to oral administration of bacteria have been detected in the saliva of animals and humans^{44, 45}, suggesting their importance as a key factor in maintaining the balance of the oral microflora⁴⁶. IgA1 was previously reported to be more abundant in older individuals than younger⁴⁷, consistent with our results (Table 3; spectral counts, group 1 = 1870; group 2 = 2320). Changes in the abundance patterns of immune related proteins highlight the complexity of the immunoprotection system. Such changes in abundance are hypothesized to occur in response to alterations in the oral microbiota with age, leading to a change in the host's immune response to a microbial challenge⁴⁸. Indeed, the increase in the total number of proteins in group 2 was partially due to the identification of nearly 2-fold more immune related proteins than in group 1 (Fig. 5A).

A significant number of salivary proteins with a primary role in host defense were also identified in the saliva of both the age groups. For example, antimicrobial proteins (AMPs) such as lysozyme, lactoferrin proline-rich proteins, mucins and histatins, which protect the oral cavity by limiting growth or killing bacteria directly, were common to both age groups. The abundance of antimicrobial proteins such as proline rich proteins, mucin-7 and bacterial permeability membrane proteins did not change with age, while lysozyme, lactoferrin, histatin-1, serotransferrin, mucin-5 and NGAL abundance increased in the older age group. In contrast, other antimicrobial proteins such as granulins and jacalin-like lectin decreased with age (group 1>2). In total, 33 proteins from groups 1, and 34 proteins from group 2 were identified as antimicrobial proteins. Using a pair-wise comparison between the groups, 19 (1 vs. 2) host defense proteins were differentially expressed. Of the host defense proteins, only 9 proteins were expressed in both age groups.

Changes in ovarian hormone levels during puberty, pregnancy, menstrual cycle and oral contraceptive use appear to correlate with decreases in the abundance of some antimicrobial proteins responsible for regulating the oral microflora, thereby compromising their protective role and allowing some bacteria to thrive⁴⁹. However, the older subjects (group 2) in this study are all post-menopausal and not receiving hormone replacement therapy. Consequently, changes in AMPs abundance between the young and old groups could be in response to changes in microflora that have been observed with age^{50, 51} and/or hormonal status⁴⁹. Regardless of the mechanism, our results demonstrate that the abundance of AMPs changed with age; such changes may play an important role in maintaining homeostasis of the oral microflora.

We found that there was a modest, but significant, decrease with age (Fig. 6) in Carbonic anhydrase VI (CA VI) which is responsible for maintaining pH homeostasis in the oral cavity and upper alimentary tract. This enzyme is stored in the secretory granules of the acinar cells

of the parotid gland, and its secretion in saliva follows a circadian cycle⁵². CA VI plays a significant role in preventing caries⁵³ and upper GI ulcers⁵⁴ by catalyzing the conversion of salivary bicarbonate and hydrogen ions released by the microbe to carbon dioxide and water⁵⁵. Consequently, one potential mechanism for the increased development of caries lesions in the age may be due to a decrease in the expression of CA VI in this population⁵⁶.

The abundance of pro-inflammatory molecules such as S100 family, calgranulins A and B also changed with age (Supplementary Table 3). These proteins are commonly implicated in acute-phase and chronic inflammatory responses⁵⁷. In contrast to the S100 family and the calgranulins, no age-related changes in the abundance of anti-inflammatory molecules such as interleukin 1 and heat shock protein were observed, even though these proteins play a vital role in the down regulation of an immune response. Thus, the detection of a wide range of pro- and anti-inflammatory response molecules, along with antimicrobial proteins, in saliva suggests their importance and correlates with the changing immunological needs of the oral cavity.

Alpha-enolase abundance was elevated in group 2 subjects relative to group 1. Alpha-enolase is a multifunctional glycolytic enzyme involved in a number of activities, such as growth control, hypoxia tolerance, and allergic responses. Notably, α -enolase was also identified as an autoantigen in Hashimoto encephalopathy⁵⁸ and lymphoid hypophysitis⁵⁹, and its expression was dramatically increased in the saliva of Sjögren's syndrome subjects⁶⁰. Consequently, autoantibodies produced by B cell in response to this autoantigen can potentially initiate tissue injury as a result of immune complex deposition^{61, 62}.

A range of cysteine protease inhibitors were also identified in both age groups. Cysteine protease inhibitors are present in saliva, tears and plasma⁶³ and play a key role in inflammatory and immune responses^{62, 63}. Cystatin D, cystatin SA and cystatin B were more abundant in group 2, while cystatin SN and cystatin C, proteins thought to regulate protein degradation at the site of inflammation or to prevent microbial infection⁶⁶, were more abundant in group 1. Two other enzymes involved in the maintenance of healthy tissue, cathepsin B and α 1 antitrypsin, were detected. Changes in their expression have been linked to malignant changes in breast tumor in females⁶⁷. In addition, secretion of the enzyme mediator 14-3-3 protein was significantly more abundant in group 2. 14-3-3 is involved in actin disassembly following an increase in the level of cytosolic Ca^{2+} and activation of protein kinase C⁶⁸. It also takes part in the regulation of apoptosis and the p53 signalling pathway, where p53 protects cells from stress-induced apoptosis.

In summary, this is the first high-throughput proteomic analysis of parotid saliva to document the proteins expressed in a healthy cohort of young and older adult females. The approach used in our study led to the confident identification of more than 530 proteins, including 183 proteins not previously detected in the parotid proteome¹⁸. Extensive age-associated changes in the abundance of many of these proteins were noted, especially for proteins associated with host defense mechanisms. Such changes in host defense proteins may reflect the dynamic nature of the very complex milieu of proteins found in saliva and their role in protection against oral microorganisms. In the present aging study, we controlled for gender, age, time of collection and state of menstruation (pre- and post-menopausal, groups 1 and 2, respectively). However, it is difficult to discriminate between bona fide age-related changes in salivary proteins from those induced by numerous other factors such as dietary, alcohol habits, periodontal disease, hydration and hormonal status. Consequently, this study provides an initial database of proteins to evaluate in future studies the effects of additional important factors on saliva composition, and to compare saliva from healthy and disease subjects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge Jennifer Hryhorenko for technical assistance with the western blots and Charlene Chung for saliva collection. We also thank Drs. Ignacio Sanz, Jennifer Anilok, and Gene Watson for development of the subject database and discussions during the course of this study. This work was supported in part by NIH grants DE017585 (J.E.M.) and P41 RR011823 (J.R.Y.). BL was supported by a CFFT computational fellowship BALCH05X5.

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A. **B.** **Figure 1. Fractionation and SDS-PAGE analysis of parotid saliva**

(A) Parotid saliva collected from seven subjects (group 1; 20–30 years of age) was pooled and subjected to hydrophobic charge interaction chromatography (HCIC). Saliva (76 mg protein) was applied to HCIC column (arrow 1), and then eluted with a 50mM Tris HCl pH 8.1 to 50 mM sodium acetate pH 4 (100:0) (arrow 2), followed by 50 mM Tris HCl pH 8.1: 50mM sodium acetate pH (0:100) (arrow 3). The unbound saliva was collected as 5 ml fractions in the flow through (FT, fractions 2–16) and elutions (E, fractions 17–61) were collected as 1ml fractions. Protein was detected at 214 nm. (B) SDS-PAGE analysis of pooled starting saliva (Lane ST), size marker (lane M), flow through fractions (FT, 3–16) and elution fraction (E, 18–53). Amylases and histatins are indicated by the arrows (AMY and HIS) at ~55 kDa and ~8kDa, respectively. Gels were run under reducing conditions, followed by Simple Blue

staining. Lane M is SeeBlue (Invitrogen) prestained molecular mass marker ranging from 3.5 to 260 kDa.

A. **B.**

Figure 2. Fractionation and SDS-PAGE analysis of parotid saliva

(A) Parotid saliva collected from seven subjects within 55–65 yrs was pooled and the salivary protein (56mg) was treated as described in Fig. 1 legend. The unbound saliva was collected as 5 ml fractions for flow through (FT) (2–15 and elutions (E) (16–60) were collected as 1ml fractions. Peaks were detected at 214 nm. (B) SDS-PAGE analysis of the flow through (FT, 3–15) and elution (E, 21–53). Chromatography for group 2 was performed identically as group 1.

Figure 3. Overlap of the proteins identified from pooled parotid saliva between the different age groups

Venn diagram showing the differentially expressed proteins identified per group and the number of proteins overlapping between the different groups.

Figure 4. Annotation of the number of identified parotid proteins by function

Protein annotation was obtained using UniProtKB/Swiss-Prot databases. Percentages of the total number of proteins identified from pooled parotid saliva from each age group are assigned to functional categories. Classification of proteins identified from 20–30 yrs (A), and 55–65 yrs (B) female subjects.

Figure 5. Comparison of the functional categories and number of proteins identified within each functional category in the pooled parotid saliva

(A) Pair-wise comparison of the number of differentially expressed proteins in the four most abundant functional categories between groups 1 and 2. (B) Pair-wise comparison of the number of differentially expressed proteins in seven functional categories between groups 1 and 2.

Figure 6. Validation of two representative salivary proteins by Western blot analysis
Immunoblots of pooled parotid saliva from groups 1 and 2 probed antibodies that detect (A) Carbonic anhydrase VI and (B) amylases.