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Potential biomarkers of human salivary function: a modified proteomic approach

J.D. Rudney*, **R.K. Staikov**, and **J.D. Johnson**
Department of Diagnostic and Biological Sciences, School of Dentistry, 17-252 Moos Tower, 515 Delaware St. SE, University of Minnesota, Minneapolis, MN 55455, USA

Summary

Objective—In previous studies, we defined groups of subjects with opposite salivary function. Group membership was associated with clinically-relevant outcomes. High aggregation-adherence (HAA) groups showed lower levels of caries, supragingival plaque, total streptococci, and *Tannerella forsythensis* than low high aggregation-adherence (LAA) groups. In this study, we used a proteomic approach to search for biomarkers which could be useful as risk indicators for those outcomes.

Design—Clarified resting whole saliva from each of 41 HAA and LAA subjects was separated by preparative isoelectric focusing. Fractions showing the most distinctive protein profiles were pooled into four sets (pI 3–3.5, pI 4–4.7, pI 5.7–7.7, pI 10–11.5). Each pool then was compared by SDS-PAGE. Image analysis software was used to quantify matched bands. Partial least squares analysis (PLS) was used to determine which of the 65 bands from all four pools were the best predictors of group membership, caries, total plaque, total streptococci, and *T. forsythensis* counts. Those bands were identified by mass spectroscopy (MSMS).

Results—Two bands consistently were strong predictors in separate PLS analyses of each outcome variable. In follow-up univariate analyses, those bands showed the strongest significant differences between the HAA and LAA groups. They also showed significant inverse correlations with caries and all the microbiological variables. MSMS identified those bands as statherin, and a truncated cystatin S missing the first eight N-terminal amino acids.

Conclusions—Levels of statherin and truncated cystatin S may be potential risk indicators for the development of caries and other oral diseases.

Keywords

Salivary proteins; Proteomics; Biomarkers; Cystatin S; Statherin; Dental caries; Dental biofilm; Streptococci; *Tannerella forsythensis*

Introduction

Many studies have attempted to relate individual variation in particular salivary protein concentrations to differences in measures of oral health or ecology. The results generally have been inconsistent. One reason for this problem may be that salivary proteins display considerable redundancy of function. Moreover, some proteins may modulate each others'

*Corresponding author. Tel.: +1-612-624-7199; fax: +1-612-626-2651. E-mail address: jrudney@tc.umn.edu (J.D. Rudney).

functions, or form heterotypic complexes that behave differently from their component parts (1). Our group has addressed those issues in three previous studies which focused on measures of salivary function as alternatives to direct quantification of salivary proteins. In the first study, we screened resting whole saliva from 149 subjects for variation in killing, aggregation and live and dead adherence of three species of oral bacteria (2). All functions were measured together in a single hydroxyapatite-coated microplate. A principal components analysis indicated that aggregation and adherence could be combined into a single factor accounting for most of the variation in the data. Measurements of the adherence of dead bacteria to hydroxyapatite contributed most strongly to this factor. Killing of bacteria in suspension represented a second independent parameter of salivary function. Persons below the 25th percentile (low) or above the 75th percentile (high) for each of those two principal components were selected for further analysis. Four groups with opposite function scores were defined as: low aggregation-adherence/low killing, low aggregation-adherence/high killing, high aggregation-adherence/low killing, and high aggregation-adherence/high killing. Collectively, these groups included 41 subjects out of the original population. We found that caries scores were significantly decreased in both high aggregation-adherence groups (HAA), whereas there were no differences in caries between groups that were high or low for killing of bacteria in suspension. In the second study, the 41 members of the opposite function groups were recalled for evaluation of their streptococcal flora (3). The results indicated that both HAA groups showed significantly reduced levels of supragingival biofilm DNA and total streptococci compared to the low aggregation-adherence groups (LAA). There were no differences in biofilm measurements related to killing of planktonic bacteria. The third study extended that comparison to periodontal pathogens, and showed that supragingival levels of *Tannerella forsythensis* likewise were significantly reduced in the HAA groups (4).

Collectively, our previous studies suggest that knowledge of salivary function with respect to aggregation-adherence might be useful for assessing the risk of oral microbial diseases. The ideal venue for risk assessment would be the dental office. However, the salivary function assays we have developed are too complex for routine clinical use. Chairside assays would be more practical if salivary function could be defined in terms of a few simple biomarkers. The protein constituents of saliva have good potential as biomarkers, in the sense that the technology now exists to miniaturize and standardize methods for protein quantification, such as ELISA (5,6). However, this potential can only be realized if differences in levels of salivary proteins are, in fact, informative. The purpose of this study was to use a modified proteomic approach to test the hypothesis that salivary proteins might serve as biomarkers which could be used to distinguish between persons who were high or low for aggregation-adherence in our previous measures of salivary function, oral health, and oral ecology.

Materials and methods

Strategy for identifying biomarkers - a modified proteomic approach

Proteomics was made possible by the development of three key technologies: multidimensional methods for protein separation (most typically by charge and molecular weight), mass spectrometry, as a means for rapid exact identification of separated proteins, and bioinformatic methods, which provide tools for the analysis and interpretation of results from large protein databases. Several ongoing studies are making use of those methods to identify and catalog every component of the exceedingly complex salivary proteome (7–14). Such projects necessarily are focused on comprehensive examination of saliva from relatively small numbers of subjects.

Our question required a somewhat different approach. We had available to us a database containing information on the salivary function, clinical status, and microbiological status of the 41 subjects in the HAA and LAA groups, plus stored saliva samples from those same

individuals. In order to look for biomarkers associated with those parameters, we needed a cost-effective means of assessing variation in the salivary proteomes of all those subjects. Two-dimensional gel electrophoresis is probably the most common approach to protein separation for proteomic studies. Overlay-type software is available for comparison of two-dimensional gels from two different experimental conditions. The number of possible parings within a group of subjects can be estimated as n(n−1)/2 (15). With 41 subjects, the number of possible pairs equals 820. Overlay analysis clearly is not practical with so many pairs to compare. As an alternative, we developed a stepwise process, in which preparative isoelectric focusing first was used to separate the proteins in a saliva sample into 20 fractions based on charge. Those fractions were compared side by side in SDS-PAGE gels for preliminary studies, to determine whether it was reasonable to reduce the number of fractions by pooling ones that appeared very similar to each other. The 41 samples then were fractioned and the fractions from each subject pooled into four sets: acidic (pI 3–3.5), moderately acidic (pI 4–4.7), neutral (pI 5.7–7.7), and basic (pI 10–11.5), as described below. For each set, the pools from each subject were run side by side in SDS-PAGE gels. This allowed us to match and quantify bands for each set in 41 subjects at the same time. Our goal was to find potential biomarkers, and not to identify every protein in these samples. Accordingly, mass spectrophotometric analysis was restricted to bands which showed strong statistical associations with the functional, clinical, and microbiological parameters in our database.

Collection and processing of saliva samples

The University of Minnesota Institutional Review Board approved all procedures involving human subjects and informed consent was obtained from all participants. Longitudinal studies have shown that salivary protein concentrations remain relatively consistent over long periods of time (1). The 41 members of the HAA (n = 18) and LAA (n = 23) groups were recalled for a second round of resting whole saliva collection within one year of the original screening study. Subjects were instructed not to consume food or beverages except water for 1 hour before saliva collection, which was done in the morning between 10:00 AM. and 12:00 PM. Each subject was asked to collect 8 ml of saliva by expectoration in polypropylene tubes on ice. Immediately after collection, the saliva sample was centrifuged at 27,000 x g for 15 min at 4° C to remove bacteria, exfoliated epithelial cells and debris.

Preparative liquid-phase isoelectric focusing

A fresh sample of whole saliva supernatant (5.5 ml) was loaded into the middle seven compartments of a Rotofor® preparative isoelectric focusing system (Bio-Rad, Hercules, CA). The rest of the compartments received 14 ml of ampholyte solution. The final concentration of the ampholytes was 0.8%. The Rotofor® was run for 4 hours at 12 W and 4°C.

At the end of each run all the fractions were collected quickly (within 10–15 seconds) and their pH was measured to insure correct distribution of the pH gradient between fractions. Ampholytes were removed from each fraction by four rounds of centrifugation at 8,000 x g for 1 hour through Microcon™ (Millipore, Billerica, MA) filters with a molecular weight cut-off of 3,000 Da. We used phosphate buffered saline (pH = 7.2) to reconstitute the samples between each centrifugation step. Samples were recovered by reversing the filters followed by centrifugation at 3,000 g for 15 minutes. Nu-PAGE™(Invitrogen, Carlsbad, CA) sample buffer (25 μl) was added to the retentate from each fraction and the samples were stored at −20° C until needed.

SDS-PAGE

Proteins in each pI pool were separated by molecular weight with a Novex™ Nu-PAGE ™ mini-gel electrophoresis system (Invitrogen, Carlsbad, CA), in combination with a ThermoFlow™ apparatus for temperature control (Invitrogen). The best results were obtained

with pre-cast Novex™ 4–12% Bis-Tris gels and MES running buffer under constant voltage of 190 V for 50 min at 24° C.

At the end of the run, the gels were stained with Coomassie Brilliant Blue R-250, which was selected, because it allows for quick identification of proline-rich proteins. These proteins stain pink-violet with R-250 in the absence of organic solvents from the de-staining solution. The rest of the salivary proteins stain blue.

Western blots

Western blots were run in preliminary experiments, to confirm that salivary proteins of known pI and molecular weight were present in their expected locations. In different experiments we probed blots with the following primary antibodies: rabbit anti-human MG2, rabbit anti-human lactoferrin, rabbit anti-human lysozyme, rabbit anti-human IgA alpha chain, rabbit anti-human amylase, rabbit anti-human albumin, sheep anti-human lactoperoxidase, and mouse anti-human secretory component. The anti-MG2 antibody was kindly provided by Dr. Robert Troxler (Boston University School of Dentistry, Boston, MA). All the other antibodies were purchased from Sigma-Aldrich (St. Louis, MO), with the exception of anti-lactoperoxidase (Biogenesis, Sandown, NH) and anti-lysozyme (DAKO, Glostrup, Denmark).

Design of protein separations

Saliva from one subject per day was processed with the Rotofor® system according to the procedure described above, until a complete set of frozen pI pools had been obtained for each of the 41 recalled subjects. We then began running SDS-PAGE comparisons for the basic (pI 10–11.5) pool, followed by the acidic (pI 3–3.5), neutral (pI 5.7–7.7), and moderately acidic (pI 4–4.7) pools. Molecular weight standards were run in the first, last, and central wells of each gel. Novex™ Mark 12 molecular weight marker (Invitrogen) was used to standardize the basic, acidic, and neutral pools, whereas the Novex™ SeeBlue® Plus2 marker set (Invitrogen) was used for the moderately acidic pool. Samples were reduced with 25 mM dithiothreitol (preliminary experiments with the basic pool had shown no difference in band patterns with and without dithiothreitol, so samples for that pool were not reduced). In order to preserve the quantitative information provided by differences in band density, we deliberately chose not to standardize protein concentrations across samples. For each pI pool, each sample was run in three different gels.

Image acquisition and processing

Gels were scanned with a GS-700 Imaging Densitometer (Bio-Rad) using a green filter (520–570 nm), which provided the best resolution for gels stained with Coomassie R-250. After image acquisition, the gels were dried and preserved for further studies.

Quantity One™ (Bio-Rad) gel analysis software was used to define lanes and bands on the gels, and to quantify each band's optical density. Lane-based background subtraction was applied and the bands were identified automatically by the software based on pre-set parameters for sensitivity and band width. The gel images then were edited in order to remove artifacts (false positive bands) and define any bands that were missed by the software (false negative bands). The lanes containing molecular weight markers were identified as standard lanes. The software used those lanes to calculate the molecular weight of the unknown bands, and matched bands of similar molecular weight across all the lanes within a tolerance of 4% of lane height. Band density was quantified as average optical density (AOD). The AOD is the sum of the density of each horizontal row of pixels within a band, divided by the number of rows in that band. Bands that were missing from any particular sample were assigned AOD values of zero, so that each band would have a complete distribution of AOD scores for all 41 subjects.

Statistical approach for identification of potential biomarkers

Because each sample was run in three different gels, the AOD for each band in a sample was expressed as the mean of 3 replicates. Mean AOD then was transformed to log(1+ mean AOD), to correct for a high degree of skewness in the distributions of mean AOD scores across subjects. Partial least squares analysis (PLS) was used to determine which of 65 distinct bands from all four pools were the best predictors of HAA or LAA group membership, occlusal caries, total biofilm DNA, total streptococci, and *T. forsythensis* counts (as recorded in our database; all microbiological measurements were obtained from buccal molar surfaces within one year of the collection of these saliva samples). PLS is a data simplification technique which is uniquely suitable in situations where variables are highly correlated, and the number of variables is greater than the number of subjects. That approach has previously been used to determine whether allelic variation in salivary acidic proline rich proteins could be used to predict caries scores (16). In this case, a separate PLS model was run for each outcome variable listed above. Each analysis included seven cross-validation runs, in which half of the samples were randomly selected and used to predict outcomes for the other half. Each cross-validation run yielded values indicating the relative importance of each band to the prediction. Those values are called VIP scores in PLS. VIP scores were averaged across the cross-validation runs, and the average VIP scores for each outcome in turn were averaged. Bands with a grand mean VIP greater than 0.8 were selected for closer examination of their suitability as biomarkers (16). This was done by using graphical analysis to check for influential outliers, and univariate t-tests or Pearson correlation coefficients to confirm associations between mean AOD scores and outcome variables. All data analysis (including PLS) was done using Statistica 6 software (Statsoft, Tulsa, OK).

Mass spectrometry

Dried gels containing high AOD examples of two bands considered to have the strongest potential as biomarkers were sent to the University of Minnesota Proteome Analysis Core Facility. There, the gels were rehydrated, and the bands of interest were removed with an automated spot cutter. Peptides were generated by in-gel digestion with trypsin or chymotrypsin, and desalted using reverse-phase chromatography (ZipTip, Millipore, Billerica, MA). Peptides then were sent to the University of Minnesota Center for Mass Spectrometry for analysis by MALDI-TOF with a QStar XL mass spectrometer. Peptide mass data were screened against all protein sequences in Genbank and likely proteins were identified. Initial protein identifications were confirmed by a second round of mass spectrometry to sequence component peptides (MS-MS).

Results

Preliminary studies

Fig. 1 shows all 20 pI fractions from a single subject, run side-by-side in paired SDS-PAGE gels. The overall appearance of the protein pattern is quite similar to published images of saliva separations in two-dimensional gels, except that the individual proteins are seen as bands instead of spots. Coomassie R-250 metachromatic staining patterns and western blots indicated that known salivary proteins were in their expected location, based on pI and molecular weight (not shown). Isoforms of many proteins were distributed across multiple fractions. For example the glycosylated and unglycosylated variants of amylase were present across the pI range from 10.0 to 4.0, although they were most plentiful in the neutral range (Fig, 1, and western blot not shown). Some adjacent fractions appeared to be virtually identical in terms of band positions, with relatively slight quantitative variations between them (Fig 1). We reasoned that it would be most informative to compare fractions that differed substantially. To avoid missing any bands that might be present in only a single fraction, we decided to pool fractions into the four

major subgroups shown in Fig. 1, for subsequent comparisons of the 41 subjects by SDS-PAGE.

Defining potential protein biomarkers by PLS

The band- matching analysis of the SDS-PAGE gels resulted in the identification of 65 distinct bands. A database was created in which each of 65 columns represented a band, and the 41 rows represented the mean AOD values observed for that band in each subject. The quantitative data for the 65 bands then were used as predictor variables in a series of cross-validated PLS analyses with group membership (HAA or LAA), occlusal caries, total biofilm DNA, total streptococci, or *T. forsythensis* counts as the outcome variables.

Table 1 lists all those bands which had a mean VIP score of ≥ 0.80 (across seven cross-validation runs) for any of the five outcome variables. For most of those bands, a high VIP score was observed for only one or two outcomes. However, four bands showed consistently high VIP scores across multiple outcomes, with grand mean VIP scores ≥ 0.80. Accordingly, bands B2, MAR9, MAR10, and NR12 were selected for further analysis.

Univariate and bivariate analyses

VIP scores can be inflated by outliers. To detect outlier effects, we examined plots of each of those four bands against each of the five outcome variables. A single outlier in the LAA group proved to be responsible for the high VIP scores for band B2 (Fig. 2A). The NR12 band in fact was missing from all but 8 subjects. All of the subjects who had that band were members of the LAA group, and this accounted for the high VIP score. However, the NR12-positive subjects represented only 35% of the LAA subjects. The presence or absence of NR12 thus could not be used to define group membership (Fig. 2B). Outlier effects were similarly evident in bivariate scatter plots of B2 and NR12 against the other outcome variables (not shown).

By contrast, the MAR9 and MAR10 bands both had broad continuous distributions. In each case, the majority of subjects with high values for those bands were members of the HAA groups. Those group differences were significant by univariate t-tests (Fig. 2C,D). Univariate t-tests were run comparing the HAA and LAA groups for each of the 65 bands, and the p values for MAR9 and MAR10 were the lowest (not shown). The AOD values for MAR9 and MAR10 showed significant inverse correlations with occlusal caries, and scatter plots indicated that HAA subjects with AOD values greater than 0.05 had lower caries scores (Fig. 3A,B). A similar pattern of significant inverse correlations was observed for buccal molar biofilm DNA, where HAA subjects with AOD values greater than 0.05 had the lowest amounts of biofilm (Fig. 3C,D). Buccal molar counts of total streptococci showed essentially the same pattern (Fig. 3E,F). Thirty-nine percent of all subjects had zero counts for buccal molar *T. forsythensis*, but the majority of those persons were HAA subjects with AOD scores greater than 0.04 for MAR9 and MAR10 (Fig. 3G,H).

Mass spectroscopy

MS-MS identified the MAR9 protein as a variant form of salivary cystatin S, which was missing the first eight N-terminal amino acids. Digestion with chymotrypsin was necessary to identify the MAR10 protein, which proved to be salivary statherin (Fig. 4).

Discussion

Statherin and the cystatin S – (AA1-8) variant were the best discriminators between the LAA and HAA groups. They likewise were the most consistent predictors of occlusal caries, and supragingival levels of total biofilm DNA, total streptococci, and *T. forsythensis*. Those

associations may or may not indicate that there is a direct molecular relationship between the functions of those proteins and salivary antimicrobial functions.

Like almost all salivary proteins, statherin appears to be multi-functional. A wide range of potential functions have been demonstrated by *in vitro* experiments. The first to be reported was the inhibition of precipitation in supersaturated solutions of calcium (17). This property is common to a number of otherwise heterogenous salivary proteins which display a negatively charged N-terminal with two phosphoserines. However, statherin appears to be the most potent inhibitor of precipitation. Given its strong calcium-binding activity, it is not surprising that statherin has been found to be a plentiful component of the enamel pellicle, both *in vitro* and *in vivo* (14,18–20). Statherin in pellicle is distributed close to the enamel surface (19), which may be related to the high levels of lubricity that were demonstrated with purified statherin in an artificial mouth system (21). However, a recent study has shown that a statherin- and calcium-rich layer also forms at the air-liquid interface of salivary films *in vitro* (22). Because of these observed properties, statherin is thought to be a major regulator of mineralization in the mouth. This might contribute to our present findings regarding caries, if one assumes that higher levels of statherin might be associated with a higher rate of remineralization. However, it is less clear why there should be relationships between higher levels of statherin and lower levels of biofilm microbes.

In common with other pellicle proteins, the binding of the N-terminal of statherin to hydroxyapatite reveals C-terminal epitopes that may serve as binding sites for oral bacteria. *In vitro* experiments suggest that statherin may offer binding sites to more pathogenic variants of *Actinomyces* species, as well as other potential pathogens such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Candida albicans* (23–26). However, in our *in vivo* study, statherin might be said to inhibit bacterial adhesion, based on its observed inverse relationship with total supragingival biofilm, total streptococci, and *T. forsythensis* (*P. gingivalis* could not be detected at any site that was sampled in this young adult population). Interestingly, two *in vitro* studies have suggested that statherin may competitively inhibit *Streptococcus mutans* binding to other salivary proteins (27,28).

An alternative possibility is that statherin might exert a direct antimicrobial effect. We have not been able to find any published reports in which such an hypothesis was tested. The amino acid sequence of statherin does not suggest any obvious mechanism of antimicrobial action. However, the possibility cannot be ruled out, at present. Statherin is known to form heterotypic complexes with other salivary proteins (29), and it might be that high levels of statherin promote the formation of complexes that expose oral bacteria to antimicrobial effects of partner proteins. We were unable to address this question directly, since such complexes are too large to enter the SDS-PAGE gels used here. Intriguingly, statherin recently has been shown to be effective in promoting the adherence of the anti-candidal protein histatin 5 to hydroxyapatite (30).

Similar issues arise in discussing potential antimicrobial activities of the cystatin S –(AA1-8) variant. This truncated form of cystatin S appears to be present to varying degrees in saliva from most persons (31). Indeed, the variant was the first salivary cystatin to be identified by amino acid sequencing (32). It was not until the sequencing of the cystatin S gene that the existence of a full-sized form was deduced, and confirmed to be present in saliva (33,34). The three-dimensional structure of cystatin S indicates that the N-terminal portion of the molecule is probably exposed to the oral environment. The (AA1-8) truncated variant can be generated by *P. gingivalis* proteases, but it is likely that it can be produced by many proteases which cleave on the carboxyl side of arginine (AA8) (35).

Cystatins generally are considered to function as cysteine protease inhibitors. The inhibitory activity of cystatin S is considered to be weak in comparison to the other salivary cystatins (C,

D, SA, and SN) (36), although cystatin S was able to inhibit up to 40% of the protease activity in *P. gingivalis* culture supernatant (35). The loss of the eight N-terminal amino acids appears to have little effect on protease inhibition by cystatin S (35). For all those reasons, it is not clear whether cysteine protease inhibition makes a strong contribution to the inverse associations seen between levels of the cystatin S – (AA1-8) variant and our outcome variables.

Cystatin S can be phosphorylated at up to five sites. Non-phosphorylated, partially phosphorylated, and fully phosphorylated variants can be found within the same saliva sample (37). It is thought that the phosphorylated forms may play some role in calcium regulation and pellicle formation, although their ability to bind calcium is approximately ten-fold less than that of statherin (36,37). Two of the phosphorylation sites is are serines at AA1 and AA2. Thus, one might expect the calcium binding activity of the (AA1-8) truncated variant to be reduced relative to the intact molecule. It is not known whether this is actually the case, and it is not clear how such a reduction might contribute the inverse associations we observed.

A more intriguing possibility arises from the observation that cystatin S can inhibit the growth of *P. gingivalis*, and that effect appears to be independent of its ability to inhibit cysteine proteases (35,38). Three peptides derived from cystatin S showed inhibitory activity, with a peptide representing AA1-14 being the most active on a molar basis. The effect of all three peptides was cumulative, with no single peptide showing as much activity as the full molecule. However, since the AA1-8 peptide is readily cleaved by *P. gingivalis*, it has been suggested that it may inhibit the growth of that organism by interfering with the uptake of small peptides (38). Such an effect has not been verified experimentally, and it is not known whether it would occur with a wide range of oral bacterial species. If that were to be the case, then the level of free peptide in saliva might influence the growth and development of oral biofilm. The proteomic approach we used did not allow us to detect or measure small peptides. However, it is reasonable to suggest that higher levels of the (AA1-8) truncated cystatin S variant might be indicative of higher levels of the AA1-8 peptide. Further study will be needed to test this hypothesis.

Clearly, more information is needed on the molecular basis for the contributions of statherin and the (AA1-8) truncated cystatin S variant to variation in salivary antimicrobial functions. However, even in the absence of such information, both proteins are still potentially useful as biomarkers for risk prediction. In our population, statherin is the better discriminator. With an cutoff value of 0.03, the AOD scores distinguish between members of the LAA and HAA groups with a sensitivity of 100% and a specificity of 67%. In other words, all members of the presumably higher risk LAA group (higher caries, biofilm, streptococci, and *T. forsythensis*) are correctly identified, but 33% of the presumably lower risk HAA group are misclassified. Further studies are needed before our findings can be generalized to other populations. In that respect, two recent proteomic comparisons of caries-free and caries-susceptible subjects from Portugal are intriguing (39,40). Those studies focused on proteins which adsorbed to hydroxyapatite, and found that statherin and cystatin S levels were higher in the caries-free group (they did not look at variant forms of cystatin S).

If additional clinical studies confirm the utility of statherin and/or the (AA1-8) truncated cystatin S variant as biomarkers, then the next step will be to develop assays for those proteins which can be used in a dental office. Recent advances in point-of-care immunoassays, microfluidics, and protein chip methods suggest that this may become possible in the very near future.

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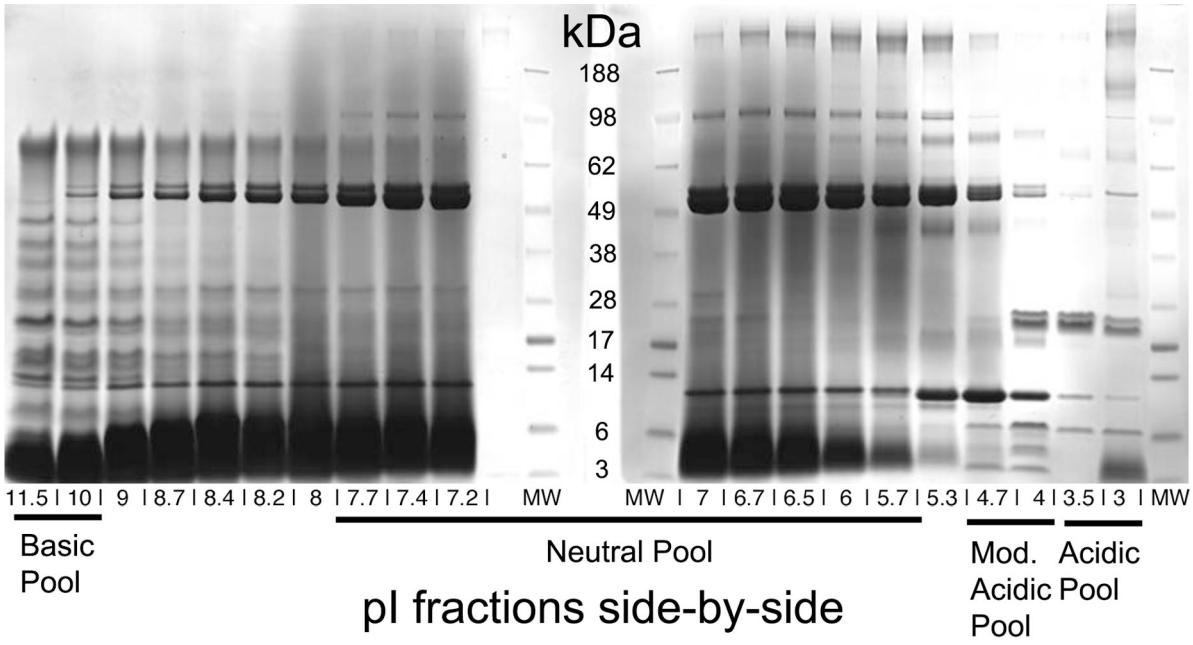

Figure 1.

All 20 pI fractions from a single subject, run side-by-side in paired SDS-PAGE gels. The horizontal dimension represents the pI fractions, from the most basic to the most acidic. The vertical dimension represents molecular weight standards in kDa (lanes containing molecular weight standards are denoted by MW). Fractions that were pooled in subsequent SDS-PAGE comparisons of multiple subjects are denoted by bold horizontal lines along the pI axis.

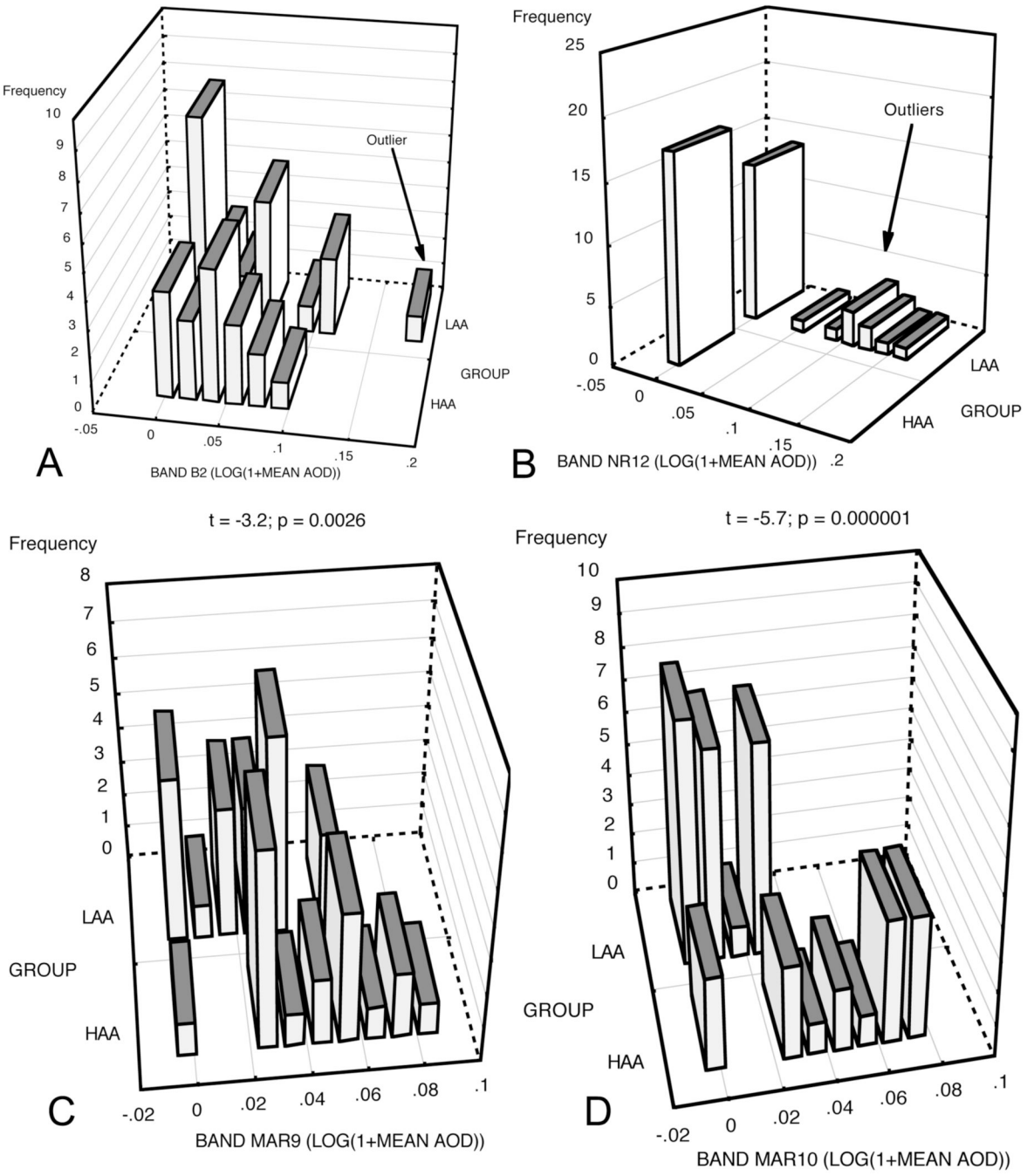

Figure 2.

Three-dimensional histograms showing the distributions of mean AOD scores for bands B2 (A), NR12 (B), MAR9 (C), and MAR10 (D), split between the LAA and HAA salivary function groups. Arrows mark outlying persons for bands B2 and NR12. Student's t-test results are given for bands MAR9 and MAR10.

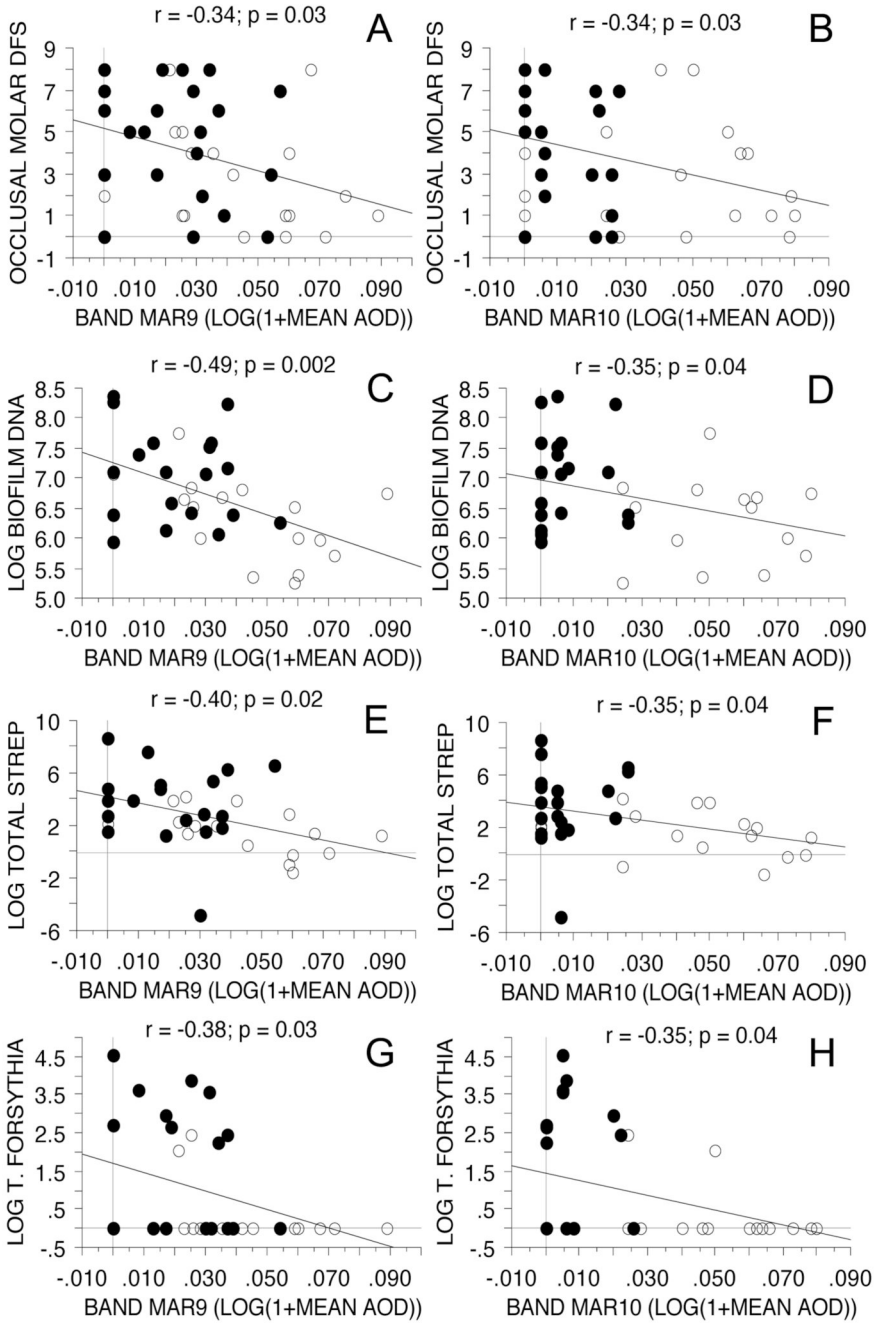

Figure 3.

Scatterplots showing regression lines and Pearson correlation coefficients for mean AOD values of bands MAR9 (left column) and MAR10 (right column) against the outcome variables of occlusal caries (A, B), buccal molar biofilm DNA (C, D), buccal molar total streptococci (E, F), and buccal molar *T. forsythensis* (G, H). Black circles denote members of the LAA group; white circles identify members of the HAA group.

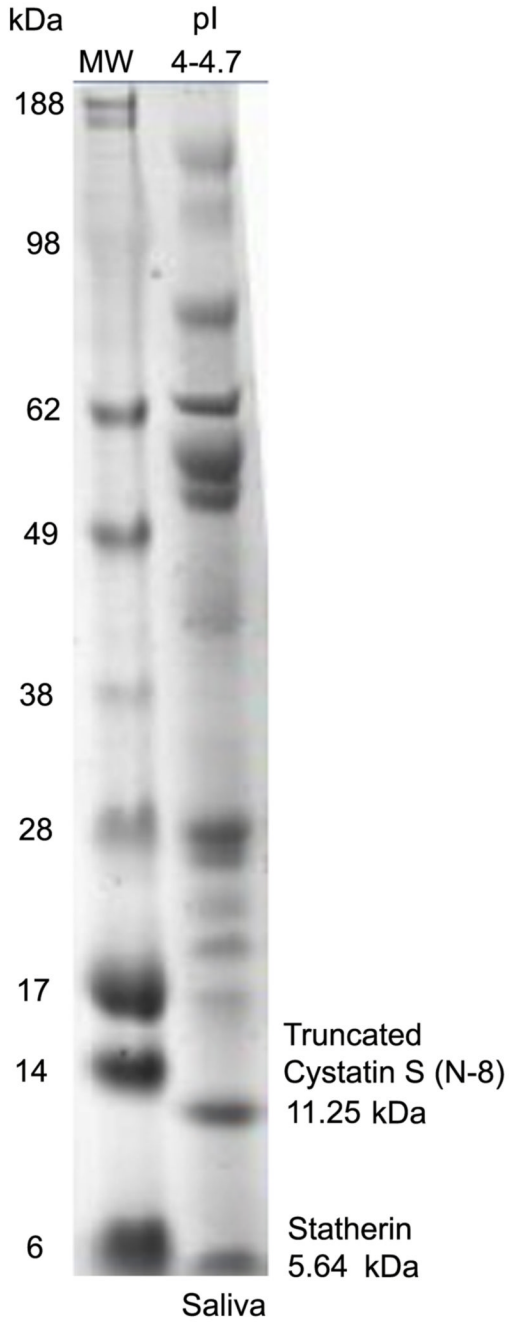

Figure 4.
The pI 4.0–4.7 pool from a single subject run in an SDS-PAGE gel, adjacent to a lane containing a molecular weight standard (MW). The location and identification of the two bands characterized by mass spectroscopy are marked as shown.

Table 1

Results of PLS analyses, showing all bands with VIP scores of 0.80 or greater (shown in boldface) for any of five outcome variables.

	Outcome variables					
Band	**Salivary function HAA/LAA**	**Occlusal molar caries**	**Buccal molar biofilm DNA**	**Buccal molar total streptococci**	**Buccal molar *T. forsythensis***	**Mean VIP**
AR4	0.54	0.63	0.56	0.56	**0.82**	0.62
B1	0.50	0.18	0.54	**0.99**	0.26	0.49
B2	0.47	**0.89**	**0.93**	**0.81**	**1.00**	**0.82**
B16	**0.96**	0.51	0.44	0.31	0.53	0.55
MAR2	**0.80**	0.71	0.52	0.55	0.58	0.63
MAR3	0.57	**0.81**	0.59	0.59	**0.87**	0.67
MAR5	0.31	0.45	0.61	**1.01**	0.44	0.56
MAR6	**0.80**	0.71	0.52	0.55	0.58	0.63
MAR7	**0.84**	**0.85**	0.43	0.40	0.59	0.62
MAR9	0.73	0.73	**0.92**	**0.91**	**0.90**	**0.84**
MAR10	**1.03**	**0.83**	0.70	0.65	**0.99**	**0.84**
NR2	**0.82**	0.27	0.54	0.55	0.65	0.57
NR3	0.26	**0.80**	0.57	**1.07**	0.86	0.71
NR12	**1.41**	0.52	**0.98**	0.50	**1.02**	**0.89**