Saliva Protein Profiling for Forensic
Subject Identification

By

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ABSTRACT

Subject identification, crucial to criminal investigations in Forensic Sciences, is in constant development due to technological advances. Generally, the identification of a perpetrator relies on trace amounts of DNA. However, sometimes the significantly low yield of DNA obtained from crime scenes and, in some cases, its degradation, prevents the generation of a reliable DNA profile suitable for subject identification. Proteins are deposited at crime scenes along with DNA and often at much higher concentrations than nucleic acids. As such, this pilot study tested the hypothesis whether salivary protein profiles could be used as fingermarks for subject identification in addition to or in lieu of DNA. Saliva samples were obtained from eleven volunteers, proteins were extracted and analyzed using tandem liquid-chromatography mass spectroscopy (LS-MS/MS). The protein functions were identified by using different publicly available and curated databases (e.g., STRING, DAVID, and Proteomicsdb). Protein profiles built with the most discriminating proteins allowed a clear separation of subjects, indicating that this approach has the potential to be used for lead generation and subject identification.

INTRODUCTION

In forensic investigations, identification of subjects (including perpetrators) involved with a crime scene is essential. DNA analysis has become integral part of this process, aiding in the identification of both victims and perpetrators of crimes. Obtaining a reliable DNA profile for identification requires a sample that contains enough intact DNA (1). However, DNA recovered from any crime scene with the purpose of subject identification has a limited chemical stability, can be easily degraded (2, 3) and, if not properly preserved, may generate an unreliable DNA profile for subject identification in forensic contexts (4). Proteins are also left at crime scenes, providing an alternate material for subject identification. Saliva is an ideal biological fluid for protein analysis because it is biologically dynamic, and its diversity depends on many factors such as diet, disease, gender, genetics, personal habits, environment, among others (5-19).

Use of saliva in forensic sciences

Historically, saliva has had many uses in forensic investigations as this biological fluid is usually present at crime scenes (5). Saliva samples can either be found as wet, whole saliva, or dried. This is significant because the compositions of dry and wet saliva samples change over time (2). A previous study determined that DNA in dry saliva samples remained stable over a year long period of storage. Under wet conditions, DNA showed acute degradation after one month. This contrasts with α-amylase, a salivary protein, which remained stable for a year in both wet and dry samples (2). Additionally, some proteins may lose functionality in dry conditions, such as RNase, an enzyme present in saliva (2). Once a saliva sample is collected, it plays a significant role in criminal investigations, traditionally acting as source of DNA, aiding in drug analysis, and providing insight into what may have occurred in crime scenes (5).

Saliva samples can be obtained directly from human skin due to biting, sucking, licking and kissing (20) or it can also be found on common items such as cigarette butts, postage stamps, envelopes, and edibles (1). Presence of saliva cannot only aid in subject identification, but it can also help to determine the sequence of events that may have occurred while the crime was being committed, i.e., what the subject drank or ate before or during the crime (21). These timelines are integral to criminal investigations because they may corroborate statements from suspects or victims.

Saliva is typically identified through presumptive and confirmatory tests. Presumptive saliva tests establish that a specific bodily fluid may be present in the sample collected at the crime scene. The most common presumptive test for saliva is the Phadebas test. This test consists of a starch-dye complex that will change color if the complex is digested by α-amylase (22). However, this test may give false positives as α-amylase is present in other body fluids, such as semen and vaginal fluid. Confirmatory tests conclusively identify the identity of the biological fluid. In the case of saliva, these tests are performed through the Rapid Stain Identification test (RSID) tests, also based on α-amylase. This test is more specific as it is antibody-based by using a lateral flow immunochromatographic strip that specifically detects the α-amylase isoform present in saliva, but not others present in other fluids. More recently the use of mass spectrometry has been proposed as a tool to identify biological matrices in crime scenes, including saliva (21). Once saliva has been identified it can then be used for DNA analysis, drug identification, and blood typing, all of which are helpful in subject identification (5).

Biological characteristics of saliva fluid: circadian and seasonal rhythms

Human saliva is a biological fluid consisting of mainly water (99%) with the rest represented by electrolytes, immunoglobulins, proteins, enzymes and nitrogenous products (23). Since 1933 (24), when the first study that investigated salivary protein concentrations in different individuals was published, there has been increasing interest in the application of salivary analyses to monitor general health (6, 8, 9, 12, 25-27), and more recently in forensic sciences (28-30). Saliva's protein diversity is a result of its predominant role in maintaining oral health. A recent review identified five main functions for saliva (23). The first one is lubrication and protection of the oral tissues. Saliva coats the oral cavity providing a barrier between the tissues and any possible irritants. Buffering action is the second essential role of saliva accomplished via the regulation of pH by bicarbonate, phosphate, urea, amphoteric proteins, and enzymes. The third one is maintenance of tooth integrity through demineralization and remineralization of enamel, both processes facilitated by minerals present within saliva. The fourth one is its antibacterial activity. Salivary glands secrete immunological and nonimmunologic agents that help to protect teeth mucosal surfaces. For example, IgA is an immunoglobulin that neutralizes viruses and stops bacterial infections. Finally, taste and digestion are functional and active roles of saliva. Saliva enhances the tasting capabilities due to its hypotonicity and zinc binding proteins, begins food digestion through the breakdown of starch by using salivary enzymes (e.g., α-amylase), and helps with lubrication of food which is essential for swallowing (23).

Saliva is a dynamic biological fluid, with many factors contributing to both its secretion and composition. It is secreted from different salivary glands and their contribution depends on the stimulation type and circadian/seasonal rhythms (31). Unstimulated, the contribution of each gland to saliva is (in decreasing order) submandibular (65%), parotid (20%), sublingual (7-8%) and other various minor glands (<10%). However, these contributions change upon stimulation

with the parotid gland accounting for >50% of salivary secretions (23). This shift has a biological relevance as submandibular and sublingual glands secrete a mix of serous and mucous secretions, the parotid gland releases serous secretions, and the minor glands discharge mucous secretions. These glands also secrete different types of proteins into saliva. For example, parotid saliva typically contains α-amylase and PRPs whereas sublingual saliva contains mainly mucins (23). Saliva secretion is triggered through a multitude of factors including chewing, tasting, and even olfactory and visual cues of food (32-34), and follows circadian and seasonal rhythms (31). In this regard, circadian low flow has been reported during sleep, whereas peaks occur during high stimulation periods (35). Yearly low flow occurs during the summer, whereas peak flows are recorded during the winter (36, 37). Circadian salivary variations affect not only flow but also the concentration of salivary components such as electrolytes and proteins (23, 36).

Saliva proteome composition: structure and function

The major families of proteins within saliva are α-amylase, PRPs, statherin, histatin, mucin, and cystatins, all impacting oral health and digestion (38). The enzyme α-amylase is required in carbohydrate digestion helping with glucose-polymer cleavage breaking down starch into smaller molecules (39). PRPs modulate calcium ions in the oral cavity, protect against toxic effects of tannins, and aid with lubrication (40). They are also known to prevent hydroxyapatite formation and help form dental-acquired pellicle (41). Statherins also support the regulation of calcium levels in the oral cavity and, in addition, they are involved in teeth mineralization (40). Histatins have anti-fungal properties (40). Mucins cover all mucosal surfaces of the body, thereby helping with mouth lubrication and with the barrier formation against possible sources of damage (42). Cystatins are a group of cysteine-containing proteins that inhibit cysteine proteases

providing protection against pathogens and controlling lysosomal cathepsins (43). Salivary glands primarily secrete “secretory type 2” cystatins (38).

Use of salivary proteome for subject identification

Protein profiling is defined as the identification and quantification of proteins within a biological sample. Salivary protein composition depends on protein expression, which in turn, depends on many factors, including, but not limited to, genetic predispositions (44) including the polymorphic nature of the major salivary protein families [e.g., PRPs are divided into acidic, basic, and glycosylated groups (40)] and post-translational modifications which may occur before secretion from the salivary glands [e.g., glycosylation, phosphorylation, exo- and endo-proteolytic cleavages (45)], disease (46-54); diet (32-34, 55), age (28, 56-59), and gender (28, 57, 59, 60). The diversity of the salivary proteome is further modified by the host- and bacteria-derived enzymes (including proteases, glycosidases, and transferases) including oxidative stress conditions originated from activation of the immune response (e.g., oxidation of methionine to methionine sulfoxide) and aggregates (61). Furthermore, as indicated above, the salivary proteome changes with the contribution of the different glands, it follows a circadian and seasonal rhythms, and contains gingival crevicular fluid, oral bacteria, epithelial cells, and neutrophils and their products.

Saliva protein profiling has previously been used in many contexts, including as a disease prognosis and diagnosis, for potential age and gender determination in forensic contexts including race/ethnicity, which all together could help to identify subjects. Many diseases change the composition of the salivary proteome, and these changes may be detected and used for not only diagnostic but also for forensic purposes (30, 46, 62, 63). The presence of such disease

biomarkers in saliva can help exclude people without the disease and include those who have the disease as possible victims or perpetrators of crimes. While this method may not directly identify the individual, it can be used as a positive reinforcement in subject identification process (30). In terms of age-dependency on salivary composition, protein concentration increases linearly with age until permanent teeth are finished developing (28, 64, 65). Others reported that salivary protein content increases until middle age, remaining constant until it decreases with advancing age (28, 57). Females have a higher protein concentration than males (28, 60). In terms of race, total salivary sIgA concentrations are significantly higher in African-American postmenopausal women than in Caucasian ones (66). Additionally, significant differences were observed between the salivary proteome from Korean populations and the integrated human salivary proteome (67). These studies suggest that the characterization of the salivary proteome would aid at determining age, gender, and race of subjects.

This complex gene-environment interaction creates subtle but unique differences between subjects likely ensuing in differences reflected as solid differences in protein profiles. Considering the variability of the salivary samples in addition to putative modifiers introduced at the collection, processing, and storage of samples, it could be surmised that protein profiles may not be suitable for subject identification. However, a study found that there is more variability in salivary proteins between individuals than in the same individual over a 5-m period (68).

These differences can then be identified through shotgun proteomics using liquid chromatography-mass spectrometry. This method can be used to identify a large variety of proteins present in complex mixtures through the mass spectrometry analysis of peptides released in proteolysis (69). Mass spectrometry of saliva samples has been used in a number of studies to obtain an in-depth analysis of salivary proteome (8, 9, 12, 15-17, 19, 63, 70-119),

further confirming the use of liquid chromatography-mass spectrometry as a powerful tool for analyzing the complexity of the salivary proteome.

However, saliva protein profiling has not yet been studied for its full potential use as a fingermark for subject identification. This study takes advantage saliva's immense diversity, and the sensitivity of the LC-MS/MS technique to determine whether saliva proteomes can serve to distinguish one subject to another with the potential to be used for subject identification in forensic contexts in lieu of DNA evidence or, more importantly, add and expand the accuracy of DNA evidence.

MATERIALS AND METHODS

Sample Collection

Eleven volunteers were recruited for this study. All subjects were females aged between 21 and 59 years old and all worked in the same building (VetMed3B School of Veterinary Medicine at UC Davis). Samples were collected on October 22, 2020 between 9:00 am-12:00 pm. To maintain anonymity, the only information collected when collecting the saliva sample was the age of the volunteer. Prior to collection of the sample, the volunteers did not eat, drink, or perform any oral hygiene routines for 15 minutes (120). They rinsed their mouths with water and they were asked to allow saliva to pool for 60 seconds (with their heads tilted back) and to deposit the unstimulated saliva (1 ml) in a sterile container provided by the researchers (121). Collection of samples was performed with informed consent as prescribed by institutional review board policies (approved by the IRB (IRBNet ID: 1544585-1, 4/17/2020).

Saliva Preparation

A modified acetone treatment was used to precipitate the proteins from the saliva samples (122-124). Four-volumes of -20°C acetone were added to each sample and left overnight in the

dark at 4˚C. Then, the samples were centrifuged at 16,000 x g for 10 minutes at 4°C. The supernatants were discarded, and the pellets were resuspended. The samples were given another two washes of -20°C acetone with a spin after each wash at 16,000 x g for 10 minutes at 4°C. The samples were dried in a vacuum for 15 minutes to eliminate acetone excess. They were resuspended in radioimmunoprecipitation assay buffer. At the time of sample collection, the COVID-19 pandemic was ongoing, and it was essential that no viral proteins were present. Due to similarities between COVID-19 and SARS-COV (125), the pre-acetone treatment would have inactivated any viral proteins that may have been present (126).

Proteomics

Samples were reduced and alkylated before digestion with LysC and digestion with porcine trypsin. Depending on sample amount, 10–100 μg of a digest prepared from each sample was analyzed by mass spectrometry. Database searching-All MS/MS samples were analyzed using X! Tandem (The GPM, thegpm.org; version X! Tandem Alanine (2017.2.1.4)). X! Tandem was set up to search the HumanFR_crap05292020_rev database (unknown version, 149657 entries) assuming the digestion enzyme trypsin. X! Tandem was searched with a fragment ion mass tolerance of 20 PPM and a parent ion tolerance of 20 PPM. Carbamidomethyl of cysteine and selenocysteine was specified in X! Tandem as a fixed modification. Glu->pyro-Glu of the *N*-terminus, ammonia-loss of the n-terminus, Gln->pyro-Glu of the *N*-terminus, deamidated of asparagine and glutamine, oxidation of methionine and tryptophan and dioxidation of methionine and tryptophan were specified in X! Tandem as variable modifications. Charge state deconvolution and deisotoping were not performed.

Criteria for protein identification-Scaffold (version Scaffold_4.11.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications.

Peptide identifications were accepted if they could be established at greater than 88.0% probability to achieve an FDR less than 0.5% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 5.0% probability to achieve an FDR less than 5.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (127) Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Protein abundance was determined through weighted spectral counting.

The STRING Database (128) was used to determine what kinds of proteins were detected as well as the interactions between the proteins present. The STRING search done was "Proteins with Values/Ranks- Functional Enrichment analysis." The gene identifier initially used in the search was the gene name determined from the LC-MS/MS data. The value used was the average percent composition. After the initial search, the UniProt Database (129) was used to fix incorrect or unknown gene names with alternate names and the STRING search was repeated. Identified pathways were given enrichment scores and false discovery rates. The enrichment scores are determined by comparing the average value of the genes within a pathway to random set of genes of the same size (128). A positive enrichment score indicates an upregulated pathway while a negative enrichment score indicates a downregulated pathway.

The protein tissue origins were determined using the DAVID Bioinformatics Database (130, 131). The gene list used in the DAVID search was composed of their official gene symbols. Similar to the STRING search, the initial DAVID search revealed gene names that were incorrect or unknown. These names were substituted with alternate gene names found in UniProt (129). The database within DAVID used for tissue expression was UP-Tissue feature.

DAVID, indicates the significance of tissue by providing a Benjamini and Hochberg adjusted p-value. The protein tissue origins were also evaluated using Proteomicsdb (132) and the top 100 most abundant proteins within the samples.

Saliva Fingermark

The percent of protein abundances were used to create each of the subjects' profiles. The first approach selected those proteins that showed the most significant variance across all subjects. The second approach selected those proteins that had the highest coefficient of variance. Additionally, protein abundance of all identified proteins was plotted against age of the subjects to determine any correlations.

RESULTS

Sample Collection and LC-MS/MS

Saliva samples were collected from 11 females (aged 21 to 59 years) following the method approved by the IRB (IRBNet ID: 1544585-1, 4/17/2020). The age distribution was 21 y (n = 1), 23 (n = 1), 25 (n = 1), 26 (n = 1), 30 (n = 1), 31 (n = 2), 33 (n = 1), 49 (n = 1), 57 (n = 1), and 59 (n = 1). The selection of using all females, all working in the same building, collecting all samples on October 20, 2020 between 9:00 am-12:00 pm, and using a specified protocol (see Methods) was to minimize confounding variables (sex, environment, circadian and seasonal rhythms), thereby making the chances of finding different profiles across subjects more stringent.

Biological Characterization of Human Salivary Proteome

Mass spectrometry analysis of saliva samples identified 973 proteins. The individual relative protein abundances were used to determine the average percent composition of each protein present. The average of each protein across subjects then was used to rank the proteins based on their abundance. The STRING Database was used to identify the gene ontology of the

973 proteins as well as to identify their interactions. The gene ontology analysis identified the Biological Process (**Figure 1**), Molecular Function (**Figure 2),** Cellular Component (**Figure 3**), local network clusters (**Figure 4**), and pathway analysis utilizing the Reactome (**Figures 5-6**) and KEGG pathway databases.

As expected for a fluid associated with immune defense, significantly upregulated biological processes were defense response to bacterium, antimicrobial humoral response, negative regulation of peptidase activity, and negative regulation of endopeptidase activity (**Figure 1**). The significantly upregulated molecular functions were endopeptidase inhibitor and regulator activities (**Figure 2**). The significantly upregulated cellular components were extracellular space or region part, cornified envelope, cytoplasmic vesicle lumen, and secretory granule lumen (**Figure 3**). The upregulated local network cluster was constituted by two units, cornified envelope and Cystatin (**Figure 4**). The upregulated pathways identified by the Reactome database were platelet degranulation, antimicrobial peptides, response to elevated platelet cytosolic Ca^{2+}, regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs), followed by platelet activation, signaling and aggregation, and hemostasis (**Figure 5**). When the pathway analysis was performed with KEGG database, the only one upregulated was salivary secretion.

As expected for compartment and processes of main intracellular localization, the downregulated cellular component was catalytic complex (**Figure 3**). The three local network clusters were cross-presentation of soluble exogenous antigens (endosomes), mesenchyme migration, and skeletal muscle myosin thick filament assembly, and proteasome (**Figure 4**). The top five downregulated Reactome pathways were CLEC7A (Dectin-1) signaling, downstream

TCR signaling, antigen processing: ubiquitination & proteasome degradation, neddylation, and activation of NF-kappaB in B cells (**Figure 6**).

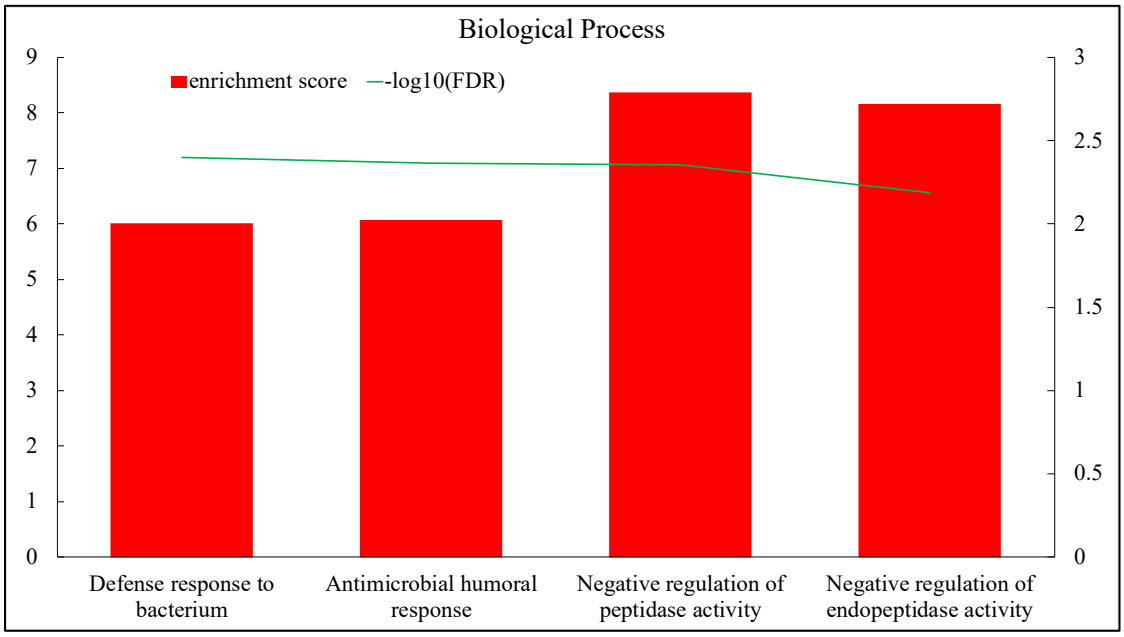

Figure 1- Enrichment scores of salivary proteins' biological processes
Data were obtained from analysis performed with the STRING database. Red is indicative of an upregulated pathway. The right and left y-axes are the -log10(FDR) and the enrichment score, respectively. Only those with a false discovery rate (FDR) below 0.05 were considered.

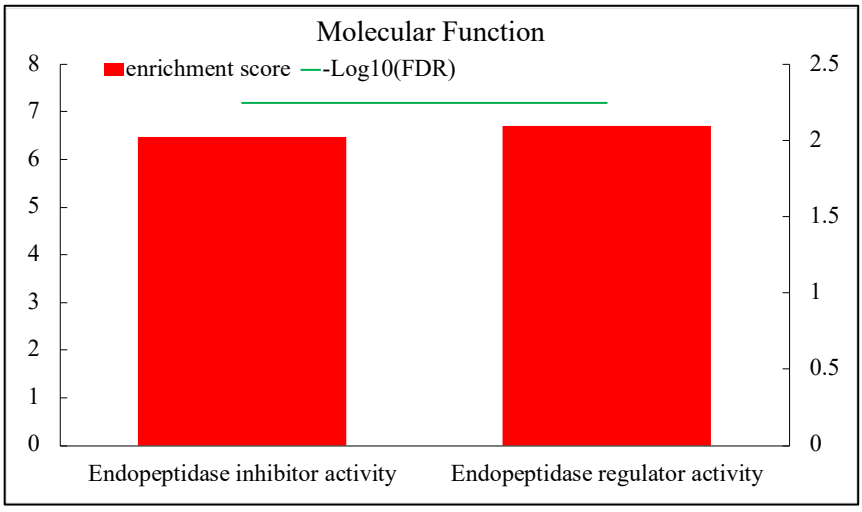

Figure 2- Enrichment scores of salivary proteins' molecular functions
Refer to **Figure 1** legend.

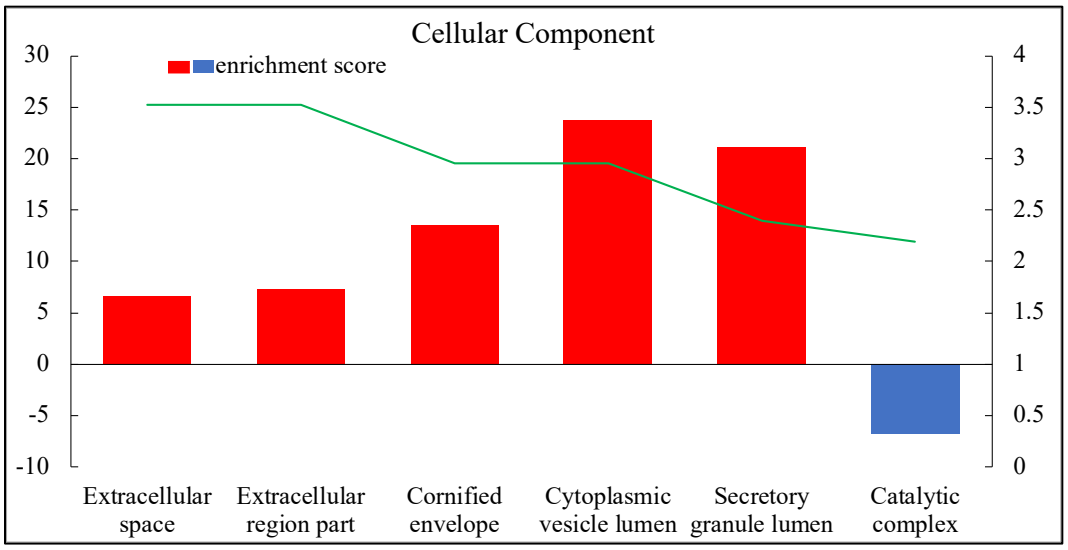

Figure 3- Enrichment scores of salivary proteins' cellular components
Refer to **Figure 1** legend. Blue is indicative of a downregulated pathway.

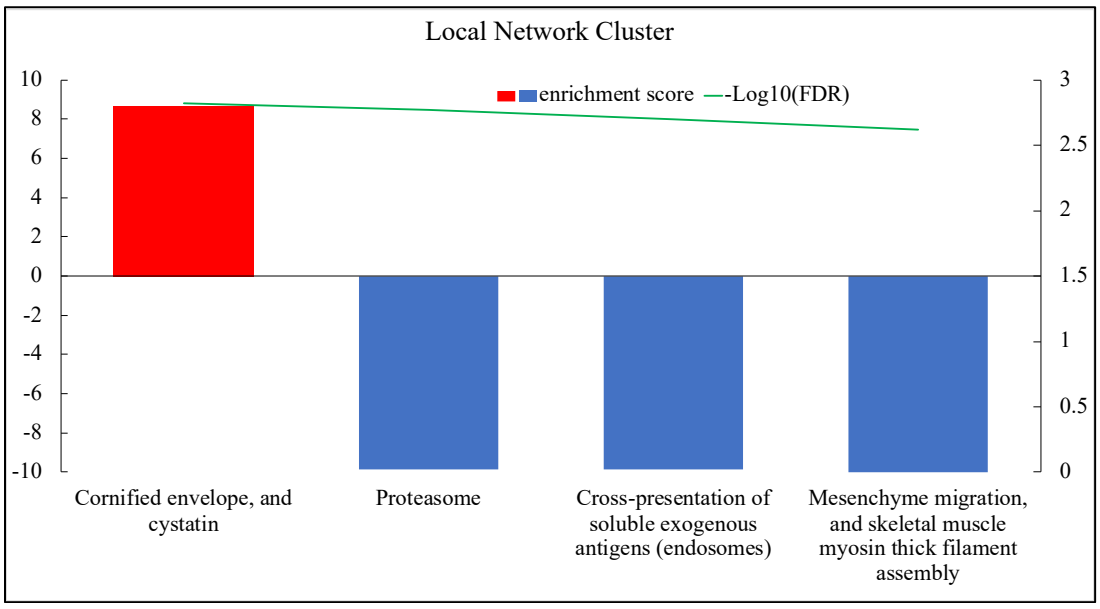

Figure 4- Enrichment scores of salivary local network clusters
Refer to **Figures 1 and 3** legends.

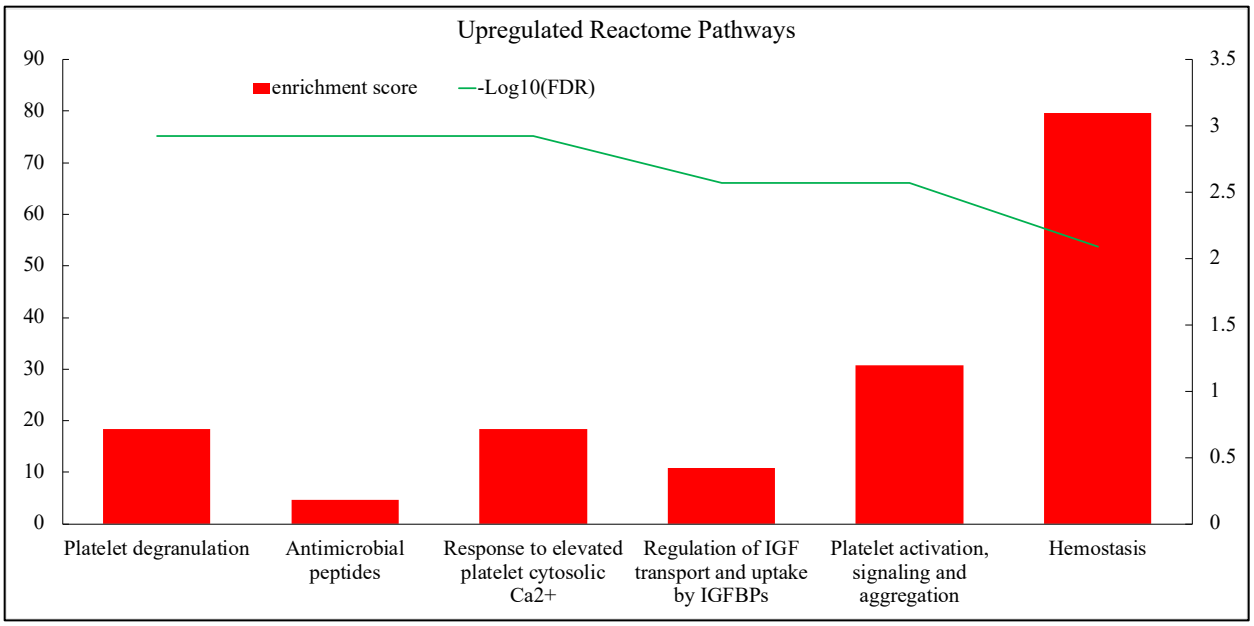

Figure 5- *Upregulated biological pathways obtained with the salivary proteome*
Refer to **Figure 1** legend. Pathway database: Reactome. Abbreviations: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding proteins.

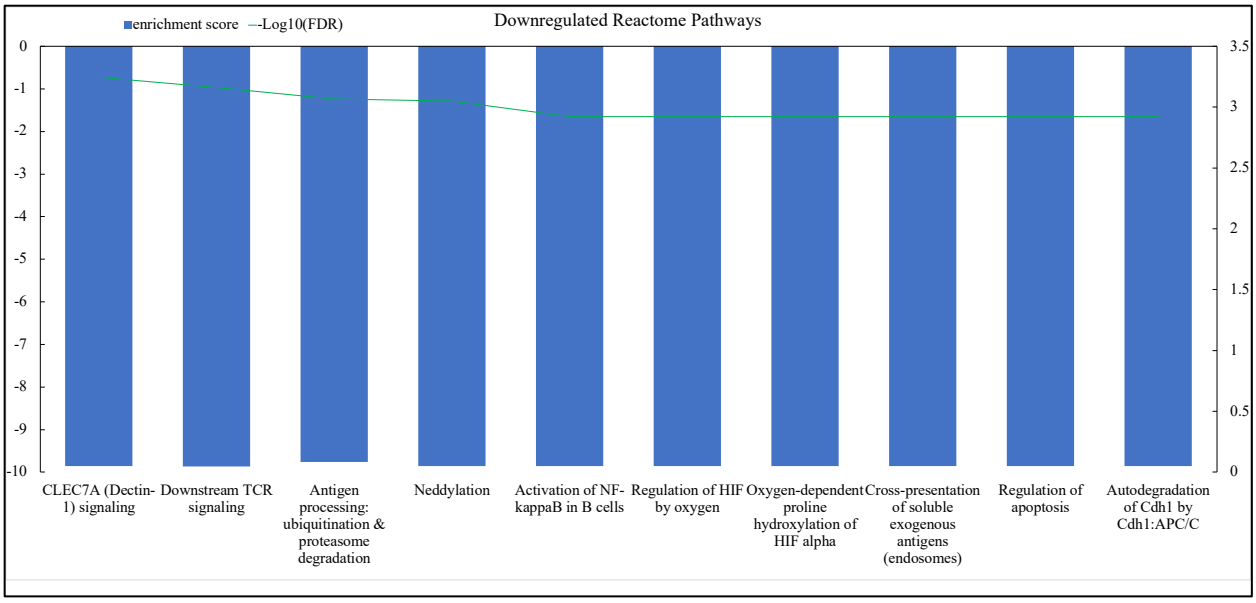

Figure 6- *Downregulated biological pathways obtained with the salivary proteome*
Refer to **Figure 5** legend. Pathway database: Reactome. Blue is indicative of a downregulated pathway. Abbreviations: TCR, t-cell receptor; HIF, hypoxia-inducible factor; Cdh1, cadherin-1; and APC/C, anaphase-promoting complex.

Tissue Expression

Tissue expression was evaluated using two databases, namely DAVID and Proteomicsdb to determine the origin of the proteins in the saliva samples as this fluid may contain acinar, various duct system, and myoepithelial cells as well as cells from tongue, palate, cheeks and pharynx. The tissues expressed according to DAVID database were epithelium, skin, keratinocyte, B-cells, tongue, and saliva (**Figure 7**). For Proteomicsdb, saliva presented the most consistent tissue expression across the analyzed proteins (**Figure 8**). Consistent with the epithelial contribution to the saliva fluid, other tissues identified were salivary gland, skin, esophagus, and tonsils.

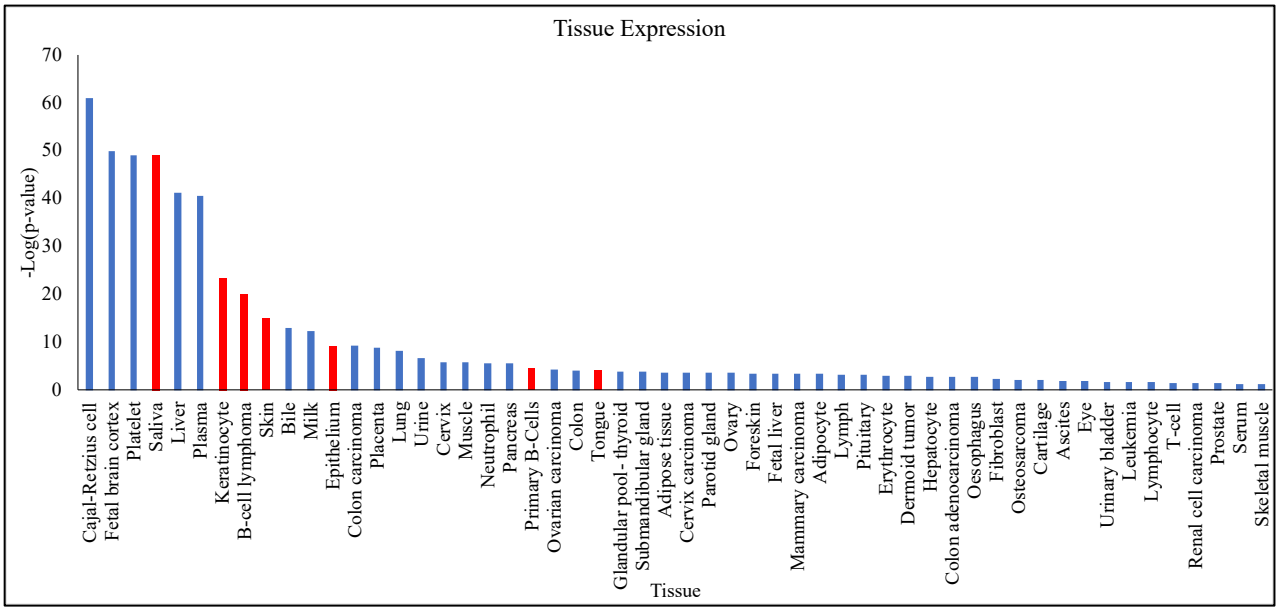

Figure 7- *DAVID Database Tissue Expression*
Only tissues with a Benjamini adjusted *p*-value <0.05 were considered. Tissues are ranked by decreasing -log(p-value).

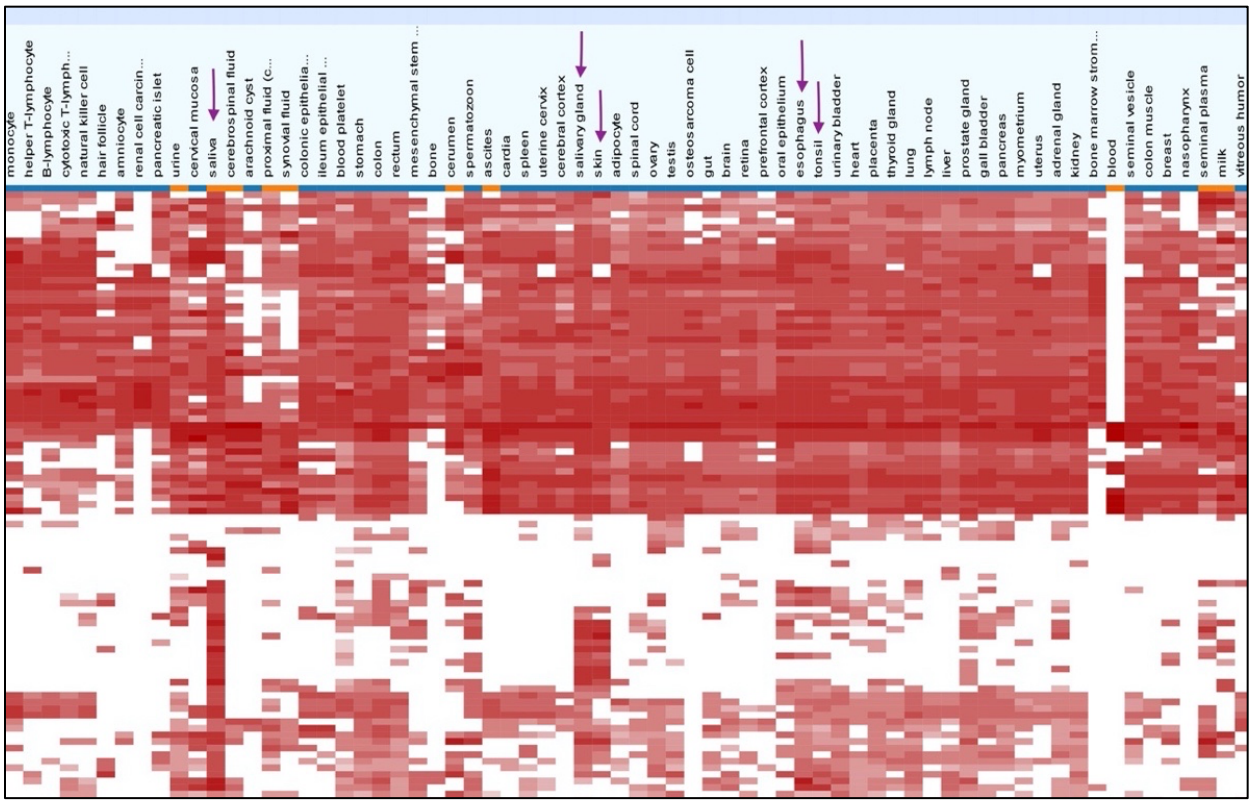

Figure 8- *Proteomicsdb Tissue Expression*
The 100 most abundant proteins were evaluated by using the Proteomicsdb. The resulting tissue expression is visualized as a heatmap. A darker shade of red indicates higher levels of tissue expression. A blue marker indicates a tissue while an orange marker indicates a fluid. Purple arrows are used to indicate consistent tissues.

Salivary Proteome for Subject Identification

This study aimed to determine whether protein profiles could be used as salivary fingermarks for subject identification. Two different methods were used to create these profiles. Only those proteins present in all eleven samples were kept. Then the variance of each protein was taken across all samples. Only those with a variance higher than 1 were kept, with 9 proteins satisfying this criterion. These proteins were α-amylase (AMY1A), immunoglobulin heavy constant alpha 1 (IGHA1), serine/threonine protein kinase (CST1), lysozyme C-2 (LYZ), polymeric immunoglobulin receptor (PIGR), albumin (ALB), mucin-5B (MUC5B), keratin, type 2 cytoskeletal 4 (KRT4), and keratin, type 1 cytoskeletal 13 (KRT13). The abundances for each

protein were then normalized to the total protein abundance of each subject to facilitate the comparison across subjects (**Figure 9**).

The second method of creating the profiles used the percent coefficient of variance, calculated as the mean of the relative abundance of each protein over its standard deviation (CV). Only those with CV >30% were kept, and 94 proteins fulfilled this criterion (**Figure 10)**. Not only the 9 proteins selected by the variance method were included here, but also the majority of the 94 belonged to defense/immune response (n = 20), metabolite interconversion enzymes (n = 11) followed by calcium-binding, transport/carriers and scaffold/adaptor proteins.

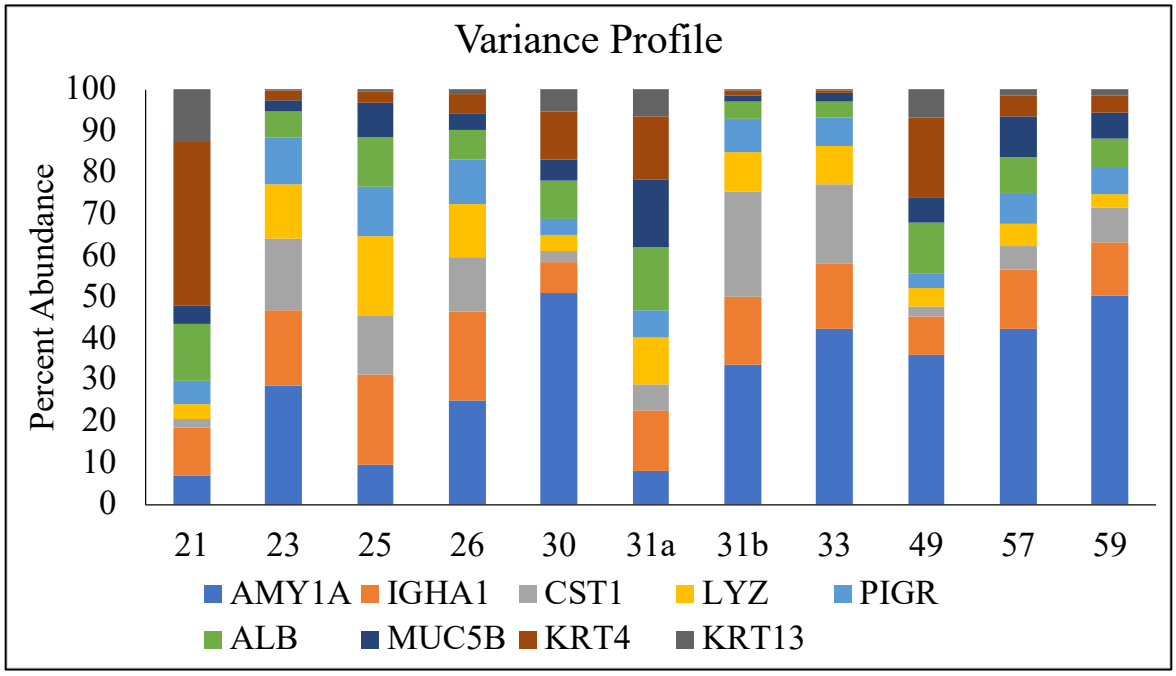

Figure 9- Salivary protein profiles selected with the variance approach
This profile is based on the 9 proteins that had variances above 1. The y-axis is abundance in percentage. The samples were ordered from the youngest to oldest subject. Each color represents a different protein.

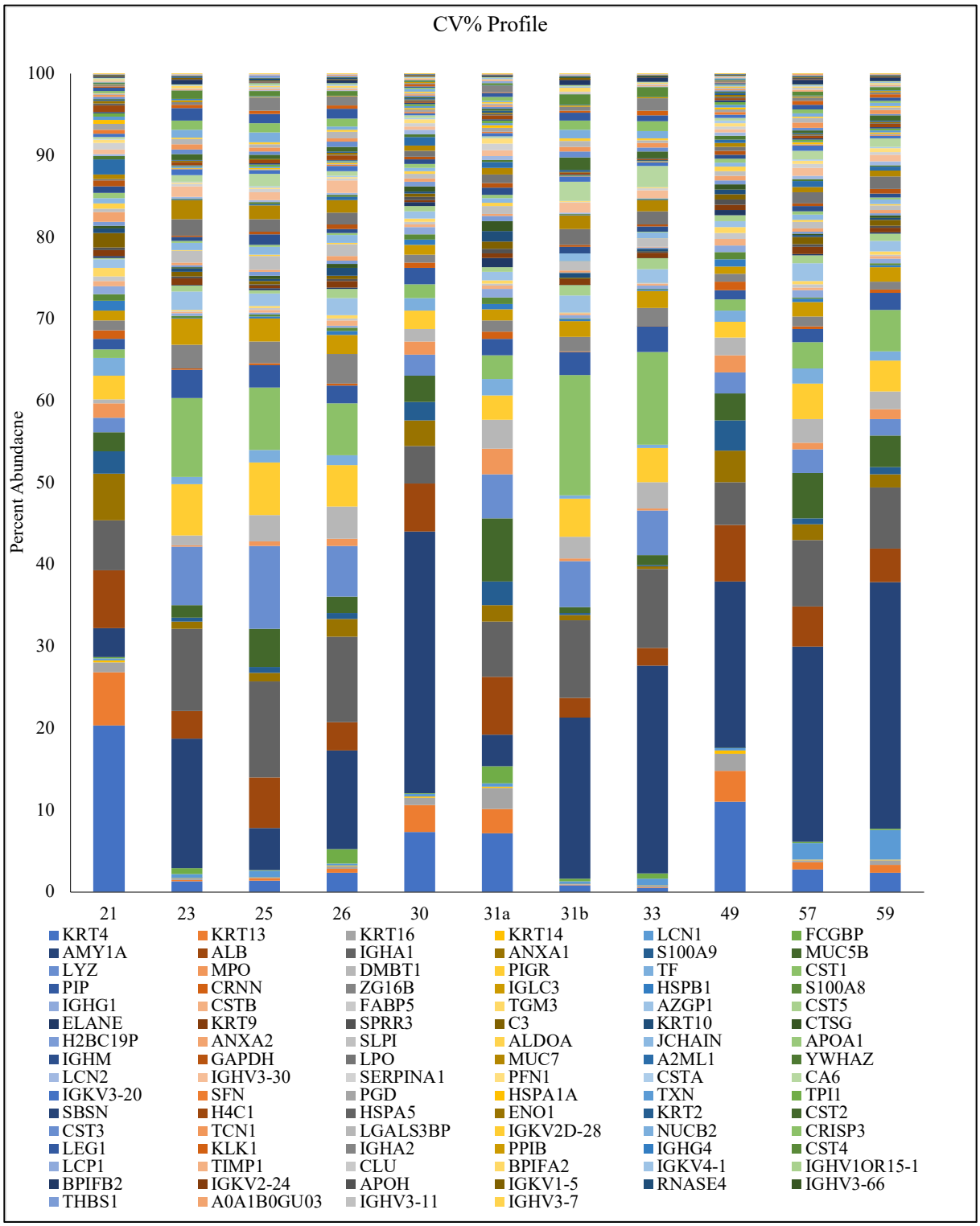

Figure 10- **Percent** *Coefficient of Variation Saliva Protein Profile*
This profile is based off the 94 proteins that had CV > 30%. The y-axis is percent abundance. The samples are ordered from youngest to oldest subject. Each color represents a different protein.

Age-dependence of Salivary Proteome

As our cohort's age ranged from 21 to 59 y old, we tested the contribution of age to the profiles. To this end, the correlation of all 973 proteins with age was analyzed. The only significant ones (Pearson's $p < 0.05$) were AMY1A, LCN1, and LCN2 (**Figure 11**), from which only LCN1 was present in the cohort of proteins identified by variance (**Figure 9**) and all three in the cohort of proteins identified by CV (**Figure 10**).

Thus, these results indicate that the age-dependent changes in protein profiles were represented by <12% of the total number of proteins identified by the variance method and <4% of the total number of proteins identified by the CV variability.

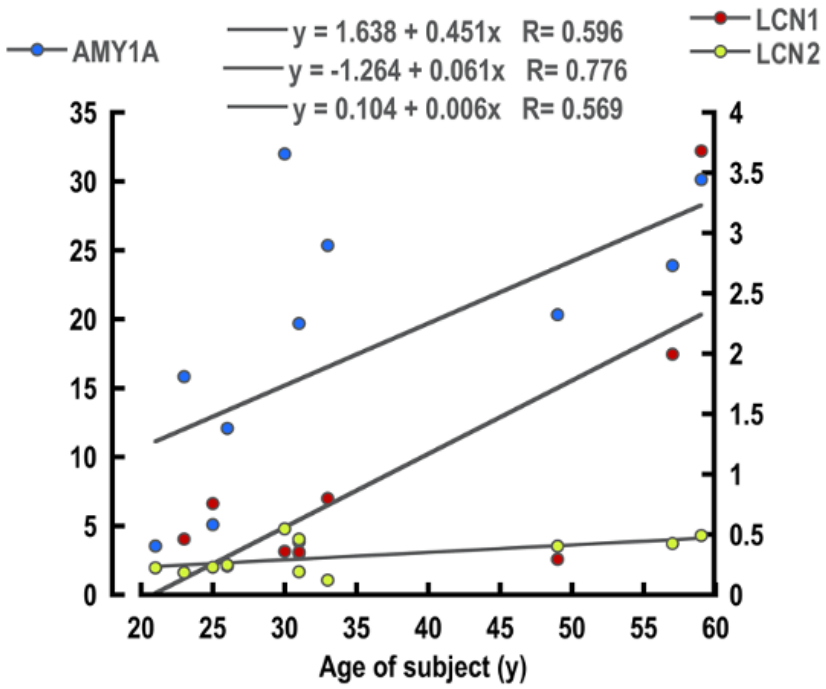

Figure 11- *Correlation of salivary protein abundance with age of subject*
Proteins significantly correlated with age (Pearson's *p*-value < 0.05). Correlation coefficients (R) and equations shown in the figure.

DISCUSSION

This pilot study aimed to determine whether salivary proteome profiles could be used for subject identification. To this end, we collected unstimulated whole saliva samples from 11 randomly selected females aged 21 to 59 y old, all working in the same building, and collected at the same time of the day (between 9:00 am and 12:00 pm) and month (October). This allowed a highly stringent setting in which differences due to sex, environment, diurnal, and seasonal circadian rhythms were minimized.

Salivary proteins were extracted, reduced, alkylated, digested with trypsin and then analyzed using LC-MS/MS, followed by GO annotation, biological pathway analysis, tissue hierarchical clustering analysis, and protein-protein interaction analysis. In total, this study identified 973 salivary proteins. This number was in between those reported by other studies utilizing mass spectrometry methods [110 to >3000; (45, 70, 133-135)] with the majority in the 1,000 to 1,300 range, consistent with our study.

As a quality control for the proteomes, the proteins identified by mass spectrometry were characterized by using their tissue of origin, gene ontology and biological pathways. This ensures that the proteins identified in this study are consistent with those that are regularly identified in saliva and consistent with saliva's function. This analysis entailed the use of several databases and algorithms provided by STRING database (128), the DAVID Bioinformatics Database (130, 131), and Proteomicsdb (132). As expected for saliva, the upregulated pathways were defense response to bacterium, antimicrobial humoral response, and the antimicrobial peptides pathway, which all matched saliva's anti-bacterial, anti-viral, and anti-fungal properties (136). Other upregulated pathways included negative regulation of peptidase activity and endopeptidase activity including the endopeptidase inhibitor activity and endopeptidase regulator

activity. These findings mirror those of a previous study, in which peptidase and hydrolase activities were enriched in whole saliva when the human salivary proteome was compared to the plasma one (137). Gene ontology analysis showed that saliva proteins were associated with 5,006 biological functions, with 439 of those associated with hydrolase and peptidase activities. This contrasts with the 362 of 5,430 biological functions in plasma that related to hydrolase and peptidase activities. The upregulation of both extracellular space and extracellular region is consistent with the presence of saliva in the oral cavity. The two clusters identified were also consistent with saliva protein-protein interactions: cornified envelope and cystatins. The cornified envelope is part of the corneocytes (type of keratinocyte), cells that cover the oral mucosa, which are important in healing oral wounds (138), and their contribution to the saliva is due to the inevitable shedding of some of these cells into the oral cavity. The other upregulated network cluster is cystatin, a major salivary protein family. Platelets were also found associated with the identified salivary proteome. Although platelets are not a natural element of saliva, they do reside within salivary glands, and some may reach the oral cavity through oral microlesions (139). The analysis performed with KEGG database indicated salivary secretion as an enriched pathway. Most importantly, both DAVID and Proteomicsdb identified saliva as the main tissue of origin for our proteome. DAVID also identified keratinocytes, epithelium, tongue, and B-cells, consistent with the identification reported by other studies (86, 137). The presence of keratinocytes and epithelium matches the finding that (on average) there are 4.3×10^5 epithelial cells in 1 ml of saliva (140). As keratinocytes and epithelium, tongue tissue and B-cells should be present as a result of either cell shedding into the saliva or as part of as production of B-cells by salivary glands (141). Proteomicsdb also identified tissue expression from the salivary gland,

esophagus, and tonsil, which are all tissues that are to be expected to be in a saliva sample due to their presence in and around the oral cavity.

Taken together, enriched, or upregulated pathways, biological functions, and compartments confirmed that the obtained proteome is representative of secreted products derived from plasma and salivary acini and conveyed the essential roles of saliva in immunity and protection. In this context, it is worth mentioning that saliva, in contrast to blood, is a non-sterile body fluid. Thus, these analyses could be used as confirmatory ones of the identity of the biological fluid as saliva.

Our pilot study aimed at testing the hypothesis whether mass-spectrometry based proteomics of saliva could be used to identify subjects in forensic investigations. Fingerprints and DNA are two of the most common methods of subject identification. However, useful fingerprints are often absent, and DNA is often degraded and left in too small a quantity to get a DNA profile that can be used for identification. This study successfully identified another possible method of subject identification utilizing salivary protein percent abundances that could be used whenever other more established methods are not helpful, to complement those or in their lieu.

Two different statistical analyses were used on the percent abundances to create the subjects' salivary proteome. The first was variance and the second was based on the variability of the coefficient of variation (CV), selecting only those proteins detected in all samples and that they had the highest discrimination power across subjects.

The profile of nine proteins generated by the variance method is relatively small for identification and when compared to the entire list of 973 identified proteins. In contrast, the CV-generated profile resulted in 94 proteins, thereby creating a more comprehensive list of proteins,

perhaps providing a greater level of certainty when identifying an individual. Furthermore, the 30% cut-off for the CV used in this study to generate salivary proteome profiles proved to be a significant discriminatory value as it is above the average CV intraday (12.8 to 15.8%) and interday variability (13 to 21.4%) of proteins from dry saliva spots obtained from parotid and submaxillar glands and analyzed performed by mass spectrometry (142).

It is known that during normal aging, oral environment and composition may change significantly, which might change the salivary proteome (28, 133, 143); however, only one out of the nine variance profile proteins and three of the 94 CV profile proteins showed a correlation with age. Overall, these results suggest that age-dependent changes did not contribute significantly to the individual protein profiles. However, 60 of the 94 proteins identified by our CV method were reported different between two age groups [young and old n=10 each, 19-24 y old and 62 to 89 y old; (133)]. Although this may seem to undermine the use of saliva proteome profiles for subject identification, the use of distinct markers for different age groups adult could provide increased uniqueness for subject identification.

Saliva samples located at crime scenes can be found as a fluid or as dry spots (2, 144). In this study, we used whole, wet saliva samples to obtain protein profiles. It may be argued that this design may not be applicable to dry saliva spots. However, comparison of our study to another one that characterized the proteome of dried saliva spots using LC-MS/MS (142) suggests that the protocol used here may be useful even when utilizing dry saliva. The study by Schulte *et al.* identified a total of 709 proteins (lower than 973 identified here) with 505 of them overlapping with the salivary proteome reported here (51.9%), indicating that most proteins identified in whole, wet saliva samples can also be detected in dry saliva spots. A more significant overlap is obtained when our proteome is corrected by the average contribution of the

parotid and submaxillar/sublingual glands (85% contribution to saliva by these glands = 827 proteins; 61.1% overlap) as in Schulte *et al*.'s study only these secretions were included from 36 males (no age reported), which as discussed earlier, differ from the proteome of whole saliva due to modifications by host- and bacteria- derived enzymes (61) further influenced by gender and age (28, 56-60).

Our proteomic analysis of whole, wet saliva revealed distinct profiles across subjects, highlighting the potential significance of this approach in forensic sciences. Future studies should include larger subject size with both sexes, collection of less abundant samples found in crime scene-like environments and collected after different periods.

CONCLUSIONS

Subject identification is essential in forensic investigations. Without identification of the victims, bystanders, and perpetrators, it is extremely difficult to understand what exactly happened as the crime occurred. For this reason, this field is constantly being investigated to establish new and more accurate methods of identification. DNA is the most common method of subject identification in forensic sciences. However, there are many instances where there is not sufficient DNA to obtain a DNA profile suitable for positive identification. Therefore, alternative, complementary methods of subject identification should be established, which this pilot study accomplished. The salivary proteome proved to be extremely diverse allowing the generation of subject-specific profiles. In a forensic investigation, the salivary protein profile obtained from a dry or wet saliva spot recovered from a crime scene could then be compared to salivary proteomes profiles of possible suspects for subject identification.

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