

# Defining Salivary Biomarkers Using Mass Spectrometry-Based Proteomics: A Systematic Review

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## Abstract

Recent advancements in mass spectrometric proteomics provide a promising result in utilizing saliva to explore biomarkers for diagnostic purposes. However, the issues of specificity or redundancy of disease-associated salivary biomarkers have not been described. This systematic review was therefore aimed to define and summarize disease-related salivary biomarkers identified by mass spectrometry proteomics. Peer-reviewed articles published through July 2009 within three databases were reviewed. Out of 243 articles, 21 studies were selected in this systematic review with conditions including Sjögren's syndrome, squamous cell carcinoma, dental caries, diabetes, breast cancer, periodontitis, gastric cancer, systemic sclerosis, oral lichen planus, bleeding oral cavity, and graft-versus-host disease. The sample size ranged from 3–41 in both diseased and control subjects, with no consensus on sample collection protocol. One hundred eighty biomarkers were identified in total; 87 upregulated, 63 downregulated, and 30 varying based on disease. Except for Sjögren's syndrome, the majority of studies with the same disease produce inconsistent biomarkers. Larger sample size and standardization of sample collection/treatment protocol may improve future studies.

## Introduction

WHOLE SALIVA is mainly composed of fluid produced by major and minor salivary glands. Major salivary glands including parotid, submandibular, and sublingual glands, are known to secrete fluid transported from serum as well as surrounding glandular tissues. This selective transportation within salivary glandular tissue is regulated by both acinar and tubular epithelial cells. Beside the secretions from salivary glands, oral mucosa, periodontium, as well as oral microflora also contribute to the final content of whole saliva. Whole saliva therefore represents a complex balance among local and systemic sources. This allows for the application of saliva in the diagnosis not only for salivary gland disorders but also for oral diseases and systemic conditions (Caporossi et al., 2010; Good et al., 2007; Hu et al., 2007a; Lee et al., 2009).

The noninvasive and simple nature of saliva collection allows for repetition and multiple collection of saliva that can potentially aid in early diagnosis, monitoring disease progression, or treatment responses with minimally trained personnel. This advantage of using saliva attracts investigators who look for an alternative form of body fluids to sim-

plify a diagnostic procedure (Giusti et al., 2007; Hu et al., 2007b; Peluso et al., 2007). In the past decade, development of mass spectrometric technologies led us to a new era in biomarker discovery that potentially will have a huge impact on future disease diagnosis and therapy. Mass spectrometry (MS) allows us to examine a salivary proteome in minute details. The presence or absence, level of expression, as well as posttranslational modifications of multiple biomarkers in a salivary proteome theoretically altered by diseases or interventions can be detected with modern MS (Caporossi et al., 2010; Good et al., 2007; Hu et al., 2007a; Lee et al., 2009).

Although there are numerous MS-based proteomic studies of serum or plasma, limited numbers of salivary proteomic studies are available. This systematic review, therefore, aims to critically review relevant clinical MS-based proteomic studies of human saliva in order to compare and contrast salivary biomarkers. In order to determine if identified biomarkers are specific to a particular disease, we compared and summarized mass spectrometry methods and identified disease-associated salivary protein biomarkers within the same group of diseases/disorders, as well as among different diseases/disorders. In addition, comparing these studies would allow for a more meaningful comparison of the results from

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different studies and provide collection of experimental protocols and disease-associated salivary biomarkers.

## Methods

To complete the review, two reviewers (S.K.A., a senior prosthodontic resident, and S.B., a prosthodontic/pharmacology faculty member) completed two independent searches using the determined databases. The search was completed through July 2009 in the following databases: PubMed (1950 to date), using the following words: [salivary (All Fields) OR "saliva" (MeSH Terms) OR "saliva" (All Fields)] AND ["proteomics" (MeSH Terms) OR "proteomics" (All Fields) OR proteomic (tw) OR "proteome: (MeSH Terms) OR "proteome" (All Fields)], EMBASE via OVID (1988 to date) using: (Proteomic.mp., exp proteomics/ or proteomics.mp., saliva analysis/ or saliva.mp. or saliva/ or saliva protein, salivary .mp., and proteome.mp. or proteome), and BIOSIS Previews via ISI Web of Science (1969 to date), ISI Citation via ISI Web of Science (1955 to date) using the following words: Saliva\* AND proteome\*. Abstracts of all articles found using the prescribed protocol were reviewed. Review articles, opinions, case reports, letters to the editors, news, and articles merely describing a technique were excluded. Only studies using human saliva were included. From the remaining research articles, only studies using MS-proteomics to compare the salivary proteomes from a disease group and a control group were selected for full text review. Only articles in the English language were included. The final articles were selected with the agreement of the first two reviewers. The third reviewer (M.B.B., a senior undergraduate predoctoral student majoring in biochemistry) was asked to review the article when there was a disagreement between the first two reviewers. Each article was then abstracted. Information on subject population, salivary sample collection and processing, mass spectrometry technique used, as well as biomarkers identified was included in the abstract.

## Results

A total of 243 articles resulting from database searches were reviewed. The database search process, excluded and included articles, as well as reasons for the exclusion are shown in Figure 1. Twenty-one articles were selected for full text review and abstracted, with information (disease, sample size, saliva type collected, selection of controls, analytical method utilized, and biomarkers identified) documented in Tables 1 and 2 and Supplementary Table 1 (Dowling et al., 2008; Fleissig et al., 2009; Giusti et al., 2007a, 2007b; Hirtz et al., 2006; Hu et al., 2007b, 2007c, 2008; Huang, 2004; Imanguli et al., 2007; Ohsiro et al., 2007; Peluso et al., 2007; Preza et al., 2009; Rao et al., 2009; Ryu et al., 2006; Streckfus et al., 2006, 2008; Vitorino et al., 2006; Wu et al., 2009a, 2009b; Yang et al., 2006). This systematic review is the first to evaluate the MS-based proteomic studies that utilized saliva to define biomarkers related to specific diseases. Results suggest that the type of saliva collected, saliva collection and handling process, proteomic techniques, and biomarker validation must be addressed to enhance the use of MS technology.

### Type of saliva collected

Variations in saliva collection can yield different proteomic profiles and, as a result, different biomarkers. From our

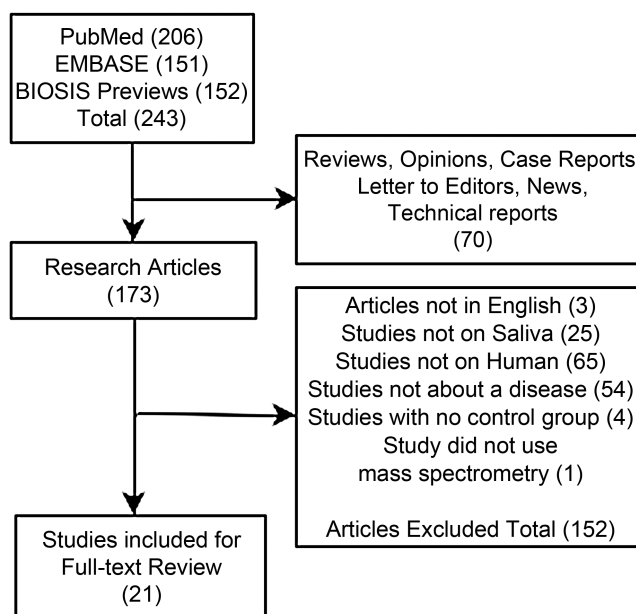


FIG. 1. A flow chart for the search results from each data base, and inclusion and exclusion criteria.

review, there are two important issues in saliva collection that may influence proteomic biomarkers; whole saliva versus individual gland saliva, and unstimulated versus stimulated saliva.

First, in assessing whole saliva versus individual gland saliva, a majority of studies chose to use whole saliva. Nineteen out of 21 studies used whole saliva samples (Table 1), representing a complex fluid from both local and systemic sources (Caporossi et al., 2010). This could have possible applications in the diagnosis of oral diseases, salivary gland disorders, and systemic conditions (Lee et al., 2009). Whole saliva collection will presumably be a composition of fluids from all major and minor salivary glands, as well as fluids from mucosal and periodontal tissues. In addition, whole saliva will also be largely influenced by oral environments, such as particular oral health problems/conditions. In studies of host immune responses in oral and systemic disease, it may be more beneficial to collect whole saliva. In addition to this, collection of whole saliva is more simple and requires minimal equipment. On the other hand, collection of individual gland saliva provides a more controlled fluid. It requires more sophisticated equipment and, therefore, is more difficult than whole saliva collection. Collection of individual gland saliva can, however, provide more specific information regarding diseases of particular salivary glands and may have little influence from the other part of the oral cavity. Only four studies in our review utilized individual gland saliva collection.

Second, in assessing the use of unstimulated or stimulated saliva, whole saliva can be stimulated or unstimulated, which differ in areas such as the ratio of contribution of major salivary glands and concentration of certain proteins, ions, and water (Ohsiro et al., 2007). Unstimulated saliva is believed to represent an equilibrated condition, having less influence from salivary glands. However, in some cases it was suggested that stimulated saliva may provide a more accurate

TABLE 1. SUMMARY OF THE FULL TEXT REVIEWED ARTICLES

Study/reference	Disease <sup>a</sup>	Sample size (diseased/control)	Saliva sample <sup>b</sup>	Technique	Number of biomarkers identified
Fleissig et al., 2009	SSI, SSII <sup>c</sup>	8, 8	UWS	2-DE (SDS-PAGE), ESI-MS/MS,	13 for SSI <sup>c</sup> 1 for SSII <sup>c</sup>
Giusti et al., 2007a	SSI	12, 12	UWS	2-DE, MALDI/TOF	9
Hu et al., 2007b	SSI	10, 10	SWS, and saliva from the parotid and submandibular/ sublingual glands	2-DE, MALDI/TOF, LC-MS/MS	16
Peluso et al., 2007	SSI,SSII	9 SSI, 9 SSII, 10 control	UWS	HPLC-ESI-MS	39
Ryu et al., 2006	SSI	41, 20	Stimulated parotid saliva	2-DE, SELDI-TOF, MALDI-TOF.	8
Preza et al., 2009	Root caries	21, 30	Stimulated parotid saliva	SDS-PAGE, LC-MS/MS	14
Huang et al., 2004	Bleeding Oral Cavity	4, 4	UWS	2-DE, MALDI/TOF, Q-TOF 2 MS/MS	10
Vitorino et al., 2006	Caries	16, 16	UWS	2-DE, MALDI-TOF	44
Streckfus et al., 2006	Breast cancer	3, 3	SWS	SELDI	6
Streckfus et al., 2008	Breast cancer	20, 10	SWS	LC-MS/MS	6
Dowling et al., 2008	Head and neck squamous cell carcinoma	8, 8	UWS	2DIGE, LC-MS	6
Hu et al., 2007c	Oral squamous cell carcinoma	20, 20	UWS	MALDI-TOF, LC-MS/MS, nanospray MS	1
Hu et al., 2008	Oral squamous cell carcinoma	16, 16—MS 48, 48—ELISA	UWS	(Shotgun proteomics reverse phase LC), LC-MS/MS, 2 DIGE	12
Ohshiro et al., 2007	Head and neck squamous carcinoma	3, 5 9,5 WB	SWS	(SAX) chromatography, SDS- PAGE and in-gel digestion, LC- MS/MS,	2
Rao et al., 2009	Type 2 DM	10 DM, 10 IFG, 10 IGT, 10 healthy	UWS	2-DLC( SCX ), LC-MS/MS.	65
Hirtz et al., 2006	Type 1 DM	8, 8	SWS	2DIGE, MALDI-TOF	23
Giusti et al., 2007b	Systemic sclerosis	15, 15	UWS	2-DE, MALDI-TOF	9
Imanguli et al., 2007	Graft versus host disease	41, 41	Stimulated submandibular/ sublingual gland saliva	2DE-DIGE,MALDI-TOF, SELDI- TOF	4
Wu et al., 2009	Generalized aggressive periodontitis	5, 5	UWS	2-DE, ESI-MS	11
Wu e al., 2009a	Gastric cancer	22, 18	UWS	MALDI-TOF	4
Yang et al., 2006	Oral lichen planus	6, 6	UWS	2-DE, MALDI-TOF	2

SSI, primary Sjögren's syndrome; SSII, secondary Sjögren's syndrome; UWS, unstimulated whole saliva; SWS, stimulated whole saliva; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; 2D-LC/MS/MS, two-dimensional liquid chromatography mass spectrometry; 2-DE, two-dimensional gel electrophoresis; 2DIGE, differential gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; ESI, electrospray ionization; SELDI-TOF, surface-enhanced laser desorption ionization time of flight; Q-TOF MS/MS, quadrupole time-of-flight mass spectrometry; SAX, strong anion-exchange array; SCX, strong cation-exchange; HPLC, high-performance liquid chromatography; DM, diabetes mellitus; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; MS, mass spectrometry; WB, Western blotting.

<sup>a</sup>Out of the 21 studies included, 5 were on Sjögren syndrome, 4 on dental caries, 7 on cancer (2 oral squamous cell carcinoma, 2 head and neck squamous cell carcinoma, 2 breast cancer, 1 gastric cancer), 2 on diabetes (1 of each for type 1 and 2), and 4 other diseases (oral lichen planus, periodontitis, systemic sclerosis, and graft-versus-host disease).

<sup>b</sup>Parotid saliva was collected using a Lashley cup (Hu et al., 2007b), Carlson-Crittenden collector (Ryu et al., 2006), or Curby cup device (Preza et al., 2009). For submandibular/sublingual glands a custom-made device or syringe aspiration from Wharton's duct was used (Hu et al., 2007b; Imanguli et al., 2007). Paraffin or gum base chewing was used primarily for stimulated whole saliva collection (Imanguli et al., 2006; Hu et al., 2007b; Ohshiro et al., 2007; Streckfus et al., 2007; Streckfus et al., 2006, 2008), whereas 2% citric acid and lemon sour candy were used to stimulate saliva collection from the specific major glands (Imanguli et al., 2007; Preza et al., 2009; Ryu et al., 2006).

<sup>c</sup>Analysis was done on eight diseased patients, six of which were diagnosed with primary Sjögren's syndrome, as well as one symptomatic patient not fulfilling the criteria completely and one who had developed follicular lymphoma.

detection of cancer biomarkers (Streckfus and Dubinsky, 2007). In some conditions where salivary flow is reduced or there is xerostomia, such as in Sjögren's syndrome or post-radiation, stimulating saliva collection may be required to obtain optimal amounts of saliva samples.

Although it is widely believed that different types of saliva (whole vs. individual gland saliva collection, and unstimulated vs. stimulated saliva) may give rise to the discovery of different biomarkers, this seems to not be the case for at least Sjögren's syndrome. Three out of five studies on Sjögren's syndrome used unstimulated whole saliva (Table 1), whereas the other two studies used stimulated saliva from individual glands. Although this collection protocol is clearly different, we found the most consistent biomarker findings among these five studies in Sjögren's syndrome (Table 2). This may have been due to the fact that Sjögren's syndrome is directly involved with salivary glands itself and may not be applicable to nonsalivary gland diseases.

#### Saliva collection and handling process

The relevance of avoiding changes in body fluid samples after sample collection is well recognized (Messana et al., 2008). Factors such as specimen collection, handling, and processing (such as the common use of protease inhibitors) affect the success of proteomic studies. Although simplicity of sample collection is often advocated as a major advantage in

using saliva as a diagnostic fluid, some precautions need to be taken in saliva collection.

First, unlike serum, saliva is susceptible to many physiological and biochemical processes, both locally and systemically (Helmerhorst and Oppenheim, 2007). Salivary contents can be altered as a result of physiological processes occurring at different points during the day, as well as due to oral stimuli (Caporossi et al., 2010). The majority of studies in our review required subjects to refrain from oral stimuli such as eating, drinking, and oral hygiene practices for varied periods of time prior to sample collection. For data to be meaningful, saliva must be collected under standardized conditions due to the presence of circadian rhythms in salivary flow rate and compositional influences.

Second, whole saliva can contain a considerable amount of shed epithelial cells, microorganisms, and remnants of food and liquid ingestion, making it essential to centrifuge whole saliva samples to remove extraneous material. This was reflected in the reviewed studies where centrifugation, with speeds varying among studies, was performed at some point within the protocol. In 12 studies in this review, the samples or the resulting supernatant after centrifugation were stored at  $-80^{\circ}\text{C}$ . Two studies had them stored at  $-70^{\circ}\text{C}$ , two at  $-20^{\circ}\text{C}$ . In five studies the samples were processed immediately. To avoid artifacts as a result of sample degradation, time elapsed between sample collection and analysis was minimized. Shortening the elapsed time to 5 min, and treating samples

TABLE 2. SUMMARY OF THE COMMONLY IDENTIFIED BIOMARKERS FOR EACH OF THE DISEASES INVESTIGATED

Disease	Biomarkers commonly identified	Techniques used in common	Techniques used in all the studies
Sjögren's syndrome (five studies) (Fleissig et al., 2009; Giusti et al., 2007a; Hu et al., 2007b; Peluso et al., 2007; Ryu et al., 2006)	Serum albumin, Actin, alpha skeletal muscle (alpha-actin-1), Ig gamma-1 chain C region, B2-microglobulin, polymeric Ig receptor, salivary amylase, lysozyme C, carbonic anhydrase VI, cystatin C, polymeric immunoglobulin receptors, prolactin-inducible protein, cystatin SN, Calgranulin A, Calgranulin B, fatty acid binding protein (epidermal).	2DE, MALDI-TOF/MS	HPLC-ESI-MS, 2D-PAGE, 2-DE, MALDI-TOF, LC-MS/MS, SELDI-TOF, 2-DE SDS-PAGE, ESI-MS/MS.
Caries (two studies) (Preza et al., 2009; Vitorino et al., 2006)	No common biomarkers	SDS-PAGE, 2-DE, MALDI-TOF	SDS-PAGE, 2-DE, MALDI-TOF, LC-MS/MS, Q-TOF 2 MS/MS
Breast cancer (two studies) (Streckfus et al., 2006, 2008)	No common biomarkers	No common technique	SELDI, LC-MS/MS
Oral or head and neck carcinoma (four studies) (Dowling et al., 2008; Hu et al., 2007c, 2008; Ohshiro et al., 2007)	S100 calcium binding protein A9 (Calgranulin B), thioredoxin.	LC-MS/MS	Reverse phase LC, MALDI-TOF, LC-MS/MS, nanospray MS, 2DIGE, LC-MS, SAX, SDS-PAGE and in gel digestion.

with TFA (trifluoroacetic acid) or other protease inhibitors prior to centrifugation and sample storage was recommended (Messana et al., 2008). Studies have indicated storage at  $-80^{\circ}\text{C}$  to be better than at  $-20^{\circ}\text{C}$ , especially after storage for prolonged times (Schipper et al., 2007). Saliva samples are composed of several proteases. These proteases potentially degrade protein biomarkers. It has been shown that leaving saliva samples for a period of time can alter proteomic profiles and change the biomarker content (Al-Tarawneh and Bencharit, 2009). Shortening the time of storage before centrifugation, fast frozen (with dried ice or liquid nitrogen), storing the samples in low temperature ( $-80^{\circ}\text{C}$ ), and avoiding multiple freeze-thaw processes, as well as consistent sample collection protocol, are known to enhance consistent proteomic analytical results and the degradation of sensitive protein biomarkers (Schipper et al., 2007).

Finally, large amounts of glycosylated proteins present in saliva often make sample handling during analysis challenging, in particular, proteomic analysis. These glycoproteins make saliva a glue-like consistency, contributing to difficulty in sample manipulation. Glycosylated biomarkers are possibly more stable and not easily degraded when compared to non-glycosylated proteins. Due to increases in salivary glycoprotein volume, chromatographic column use may provide data dense with those glycosylated species and void of other potential biomarkers (Ramachandran et al., 2006).

#### *Selection of controls*

Selection of appropriate controls is essential to avoid false identification of biomarkers due to nondisease related differences between the control and the diseased groups. Regarding selection of control groups, some of the studies did not indicate specific inclusion and exclusion criteria, whereas others followed specific protocols including: absence of clinical symptoms related to the disease in question (Fleissig et al., 2009), enrolling individuals with similar mean age and demographic characteristics to the diseased group (Giusto et al., 2007a, 2007b; Hu et al., 2007b, 2007c) using matched siblings (Imanguli et al., 2007), and negative findings in tests specific to the disease (Peluso et al., 2007; Ryu et al., 2006).

#### *Proteomic techniques*

Analysis of human saliva proteomes is inherently challenging because the salivary proteome contains a large number of proteins within an extremely wide concentration range. Initial biomarker fractionation is required for MS-based proteomic techniques. Each fraction or spot is subjected to certain mass-spectrometric technology. The resulting mass data is then used to search protein, genomic, expressed sequence tag (EST), and other species-specific databases to identify proteins present in each selected spot. By comparing samples, changes in the level of expression of individual proteins can be detected and quantified, permitting the identification of biomarkers associated with specific pathologic or physiologic states of a cell or tissue. Our review shows that there are four different techniques used (at times in combination) in biomarker screening and identification. These include 2DE, 2D-liquid chromatography/mass spectrometry (LC/MS), matrix-assisted laser desorption/ionization-time of flight/mass spectrometry (MALDI-TOF/MS), and surface enhanced laser desorption/ionization time-of-flight

mass spectrometry (SELDI-TOF/MS) (Supplementary Table 2).

2DE (two-dimensional gel electrophoresis), a combination of SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), and IEF (isoelectric focusing), is capable of separating and resolving complex protein mixtures, enabling the visualization and identification of several thousand proteins on a single gel. The drawbacks, however, of 2DE include poor gel-to-gel reproducibility, large amounts of required sample, time required, extensive labor, and low sensitivity. Our systematic review (Supplementary Table 2) suggests that 2DE is still the most popular technique for the global analysis and initial profiling of saliva prior to further fractionation and identification with other high throughput techniques. Most studies used 2DE as a first step for protein separation, followed by tandem MS (MS/MS).

The second method used is the 2D-LC/MS or the multidimensional protein identification technology (MudPIT)/shotgun and the subsequent search of large-scale databases such as SEQUEST and MASCOT. Liquid chromatography methods coupled with MS or LC-MS/MS are suitable for separation and identification of low-molecular-weight components. However, this technique still has the disadvantages of being labor intensive and provides limited information about the relative abundance of the detected proteins. Improvement of chromatographic technology allows for increased high throughput capacity of this technique.

The MALDI-TOF/MS technique is an improvement in terms of its high sensitivity for a large mass range and simplicity of interpretation of the mass spectra (Ramachandran et al., 2006). This technique was used in some studies for initial profiling prior to further identification using LC-MS techniques, whereas in other studies it has been used as the main identification technique after initial profiling with 2DE (Supplementary Table 2). In some cases the SELDI-TOF/MS technique where the sample matrix (Protein Chip) has an active role in sample purification, desorption/ionization, and protein separation] was used to quantify and reproduce results (Al-Tarawneh and Bencharit, 2009).

#### *Biomarker discovery and validation*

A biomarker is defined as a pharmacological or physiological measurement that is used to predict a toxic event; a specific molecule in the body, which has a particular feature that makes it instrumental for measuring disease progression or the effects of treatment. Biomarkers are by definition suitable to develop new diagnostic tools, alone or in combination with traditional methods (Brinkman and Wong, 2006). It is essential to choose the proper sample type, processing and handling, and proteomic techniques that will be used prior to initiation of biomarker discovery, as these techniques differ tremendously based on the intended biomarkers (Streckfus and Dubinsky, 2007). From this review, several techniques were used for biomarker discovery and quantification after the initial profiling such as two-dimensional difference gel electrophoresis mass spectrometry (2D-DIGE-MS), label free LC-MS/MS, and isobaric tagging for relative and absolute quantitation (iTRAQ) labeling prior to MS/MS quantification. In this review, some studies expressed the results of proteomic identification and quantification as a number of the differentially

expressed peaks or spots of peptides or proteins, whereas others further identified those differentially expressed biomarkers to the specific corresponding proteins (Supplementary Tables 1, 2, and 3).

## Discussion

Collecting saliva is often perceived as being distasteful by research subjects, as well as clinician researchers. Although we found that from our review the issue of compliance of subjects and researchers was not addressed in any of the selected articles, it is important to note that most salivary proteomic research has been done in academic institutes that deal with oral health, in particular, dental schools. Note also that for several of these studies, the final research goal is to seek a simple, noninvasive, and relatively inexpensive diagnostic salivary tool. These rationales are perhaps some of the best arguments for advocating salivary proteomic biomarker discovery research.

Although our application of systematic review to proteomic studies is unconventional, similar studies appear in the current literature. For example, Atiomo et al. (2009) used systematic review to describe the biomarkers for polycystic ovary syndrome. Liu et al. (2011) similarly utilized systematic review to define common biomarkers associated with Hepatocellular carcinoma. We believe that utilizing systematic review to evaluate salivary proteomic/biomarker discovery studies may allow for future improvement in this rapidly growing field and may facilitate the development of saliva as a diagnostic fluid in the future.

Ultimately, 180 differentially expressed biomarkers were identified (Supplementary Tables 1 and 3). Of these, 87 were found to be upregulated, 63 downregulated, and 30 were found to vary based upon the disease investigated. For example, Cystatin SA-III was found to be upregulated in each reviewed case of breast cancer, bleeding oral cavities, and Sjögren's syndrome (Supplementary Table 3). Its upregulation makes it a valuable biomarker for indicating instances of disease progression, but due to the nonspecificity of its expression in terms of disease type, its development into a diagnostic tool may not be the most promising.

Similarly, looking at Acidic PRP-1 1P, its expression is downregulated in each reviewed case of Sjögren's syndrome, and type 1 diabetes (Supplementary Table 3). Just as with those biomarkers continually upregulated, this biomarker's constant downregulation makes it valuable for general identification of disease progression, but lacks the desired level of specificity needed to be developed into an effective diagnostic tool.

Alpha-amylase is representative of biomarkers whose expression varies based on the disease in question, being upregulated in cases of dental carries, Sjögren's syndrome, periodontitis, and graft-versus-host disease, while being downregulated in type 1 diabetes (Supplementary Table 3). Its more closely specific directions of expression make it more applicable as a biomarker to be developed as a diagnostic tool; however, specificity issues still arise, albeit to a lesser degree.

Overall, the identified biomarkers and their expression demonstrate the potential use of a combination of significant biomarkers to structure a more complete diagnostic tool. The potential exists for combinations of identified biomarker expression, or the correlation of biomarker expression and

clinical assessments, to be utilized to achieve effective disease diagnosis. In 11 of the studies, the identified biomarkers were further validated. Immunoassays such as ELISA and Western blotting were used in the majority of the studies (Supplementary Table 2). In the case of clinical proteomics, it is likely that multiple novel candidates will be identified, meaning multiple reaction monitoring/stable isotope dilution (MRM/SID-MS) using quadruple MS may allow for greater throughput, accuracy, and sensitivity than antibody development (Rifai et al., 2006). We found that, except for Sjögren's syndrome, few biomarkers were found to be common in different studies of the same disease. This can be attributed to inconsistencies in saliva collection and processing protocols, small sample numbers, as well as differences in MS platforms (Table 2).

Analyses of normal human salivary proteomes compared with diseased proteomes should demonstrate molecular profiles that lead to disease-specific molecular biomarkers. However, this trend has not been the case in most of the salivary proteomic studies. Whole saliva represents a complex fluid as a result of contribution from both local and systemic sources. Unlike plasma, once saliva is secreted into the oral cavity, little regulation from the host can be achieved. Oral microflora and tissues also play an important role in this complexity. Changes in salivary proteomes resulting from diseases or disorders that may be measured as a significant change may not be disease/disorder-specific. There are two important issues concerning the validity of salivary proteomic analyses and the discovered biomarkers. These include (1) sample treatments and mass spectrometry analysis methods, and (2) nature of the disease.

To address the first issue, future studies must include larger samples of patients and follow a standardized protocol. Validation of novel biomarkers is of paramount value, especially when use as a diagnostic tool for personalized medicine based on these biomarkers is the final goal. Although salivary proteomics are increasingly widely used, there are several issues that must be addressed in future studies. These include a standardized protocol in saliva sample collection, unification in treatment protocol, and differences in the use of analytical methods. Future studies must also focus on the use of a standardized protocol for data analysis. Ultimately, a larger sample size is required for validation and generalization of results in future salivary proteomic studies.

Even with using the same samples, different sample treatments/collection protocols and mass spectrometry proteomic platforms can result in completely different biomarker profiles. Recent methodology reviews suggest methods that are carefully planned and standardized. This may help reduce the differences within the study and allow for a more meaningful comparison among studies (Henson and Wong, 2010; Hu et al., 2010; Loo et al., 2010; Ohshiro et al., 2007).

Several of the biomarkers listed here and detected by MS-based proteomic techniques have been discussed elsewhere in the literature to be detected with different techniques such as the use of Western blotting in detection of Psoriasis (S100A7) as a potential predictor of pulmonary involvement in systemic sclerosis (Baldini et al., 2008a,b). Lactoferrin and beta-2-microglobulin and cystatin have been shown to be biomarkers for Sjögren's syndrome using ELISA, and enzyme inhibition assays (Carpenter et al., 2000). As for breast cancer related biomarkers, C-erbB-2, and epidermal growth factor (EGF)

were investigated previously using ELISA and western blotting (Bigler et al., 2002; Brooks et al., 2008; Steckful and Bigler, 2005). Lactoferrin detection has been related to periodontitis (Komine et al., 2007), type 2 diabetes mellitus (Dodds et al., 2000), and Sjögren's syndrome both by ELISA and Western blotting (Carpenter et al., 2000). Although only a few biomarkers are confirmed by other methods, several biomarkers found in most studies were not confirmed by other analyses or by other non-MS methods.

Based on a combination of all salivary biomarkers, we can divide biomarkers into three categories: (1) biomarkers specific to a disease, (2) biomarkers that are nonspecific to a disease but seems to demonstrate an abnormal condition, and (3) biomarkers that are randomly seen that may be a result of diversities or variations between control-diseased samples, sample treatment protocols, and mass spectrometry platforms.

Aside from the fact that we are interested in variations/similarities in sample collections and analytical protocols, our rationale to include all mass spectrometry-based salivary proteomics regardless of how diverse the conditions/disease was that we want to answer two important questions. First, are there any specific salivary biomarkers for a certain condition or disease? Second, are there any salivary biomarkers that may not be specific for a disease but the changes in these particular biomarkers alarm an abnormal condition?

Although in a conventional sense, one may believe that there should be a specific biomarker for a certain disease. This may be true in many diseases, for example, particular breast cancer cells express specific proteins including C-erbB-2 and EGF (Bigler et al., 2002; Brooks et al., 2008; Streckfus and Bigler, 2005). Unfortunately, most diseases/conditions that are included in our collection (perhaps except for Sjogren's syndrome), are within themselves a collection of diseases/conditions. For example, an oral cancer even only for squamous cell carcinoma, could range from a carcinoma *in situ*, to a frank cancerous lesion to a metastasized tumor. Even worse, in caries or periodontal disease, these conditions can be caused by numerous factors, from variations in microorganisms to host responses. Our oral diseases are hardly homogeneous as one may assume.

It is interesting to examine the discrepancies in identified biomarkers in similar diseases, for example, root caries and caries. There were virtually no common biomarkers between these two studies (Preza et al., 2009; Vitorino et al., 2006) (Table 1 and Supplementary Tables 1, 2, and 3). This fact could be attributed to the following reasons. First, the investigators used different types of collected saliva and collection protocol. The root caries group used stimulated parotid saliva, whereas the caries group used unstimulated whole saliva. It is possible that whole saliva content would be different from individual gland saliva in terms of contribution from other sources in the oral cavity (see Results, Type of saliva collected). Second, the proteomic methods are clearly different. The former group used SDS-PAGE and LC-MS/MS, whereas the latter used 2D gel electrophoresis (2-DE) and MALDI-TOF/MS. Clearly results differ both in the number of identified biomarkers and the identities of the biomarkers. 2-DE will clearly give better biomarker separation (based on both charge and mass) and, therefore, yields a higher number of biomarkers (14 biomarkers in root caries study, Preza et al., 2009; and 44 in caries study, Vitorino et al., 2006). This type of difference is

also reflected in other studies. Note that in more recent studies, applications of different version of chromatography (e.g., SELDI-TOF/MS, HPLC, and other affinity chromatography) appear to provide larger amounts of biomarkers. Finally, biomarker validation remains very different in these two studies. Western blotting was used in the root caries study, whereas the caries study did not provide any secondary methods. Traditionally, antibody-based methods, including Western blotting, ELISA, and multiplex, are recommended to validate the presence of biomarkers. However, these methods require the availability of certain antibodies. It is possible that there may be no antibody available for novel biomarkers. If there are antibodies specific for a particular biomarker, they may or may not interact with the MS identified biomarkers because the antibody detection requires the presence of a specific antigenic epitope. Several recent MS based biomarker discoveries may not report a few biomarkers with confirmation from antibody-based assays but usually report most unconfirmed biomarker hits from database searches.

## Conclusions

Current literature suggests that increasing sample size and utilizing a controlled/standardized protocol can improve future salivary proteomic research. Confirmation of biomarkers should be done with more than one analytical platform. Alterations of certain salivary biomarkers, either disease-specific or disease-nonspecific, may allow a molecular insight that possibly precedes or coincides with clinical changes. Taking all this into account, examination of both disease-specific and disease-nonspecific biomarkers may, therefore, allow us to develop a salivary proteomic-based diagnostic tool. Careful clinical evaluation of particular patients and evaluation of salivary biomarkers, along with other clinical parameters, may advance our utilization of salivary diagnostics as a paramedical diagnostic tool.

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## Author Disclosure Statement

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