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Buonocore Lecture

Salivary Diagnostics

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Clinical Relevance

The past 10 years have witnessed significant advances in the science and technology of salivary diagnostics. The integration of salivary diagnostics into the clinical practice of dentistry is on the horizon to enable chair-side detection of oral and systemic disorders in the dental office.

SUMMARY

Saliva is a noninvasive and accessible biofluid that permits early detection of oral and systemic diseases. Recent scientific and technologic advances have uncovered specific salivary biomarkers for a number of clinical conditions, including cancers, autoimmune diseases, and cardiovascular disorders. The availability of highly sensitive and high-throughput assays such as microarray, mass spectrometry, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and nano-scale sensors that can measure proteins and nucleic acids are poising saliva as an emerging biofluid for translational and clinical applications. This paper will discuss development of salivary biomarkers for the detection of oral and systemic diseases and the translational application of these markers for clinical applications.

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INTRODUCTION

Current accurate diagnostic procedures available for most diseases require invasive sampling performed by trained professionals and the use of expensive, specialized machinery and equipment. The discovery that saliva contains molecular profiles that reflect diseases in the body has opened the doors to a new noninvasive diagnostic methodology: salivary diagnostics. Using saliva in early detection of diseases is quickly proving to be not only practical, noninvasive, and safe, at times it is proving more accurate than available alternatives.

Salivary diagnostics is a late comer; however, in the last decade there has been significant progress in the field. In this article, we will review the recent advances made in salivary biomarker-based diagnostics via genomic and proteomic approaches and their implications for dentistry and medicine.

SALIVA: THE BIOFLUID

Saliva is the secretions by the three major salivary glands (parotid, submandibular, and sublingual), hundreds of minor salivary glands, and gingival crevice fluid. The functions of saliva are many and, among others, include "regular" functions such as food digestion, bolus formation, lubrication, and taste facilitation, and immune functions through antimi-

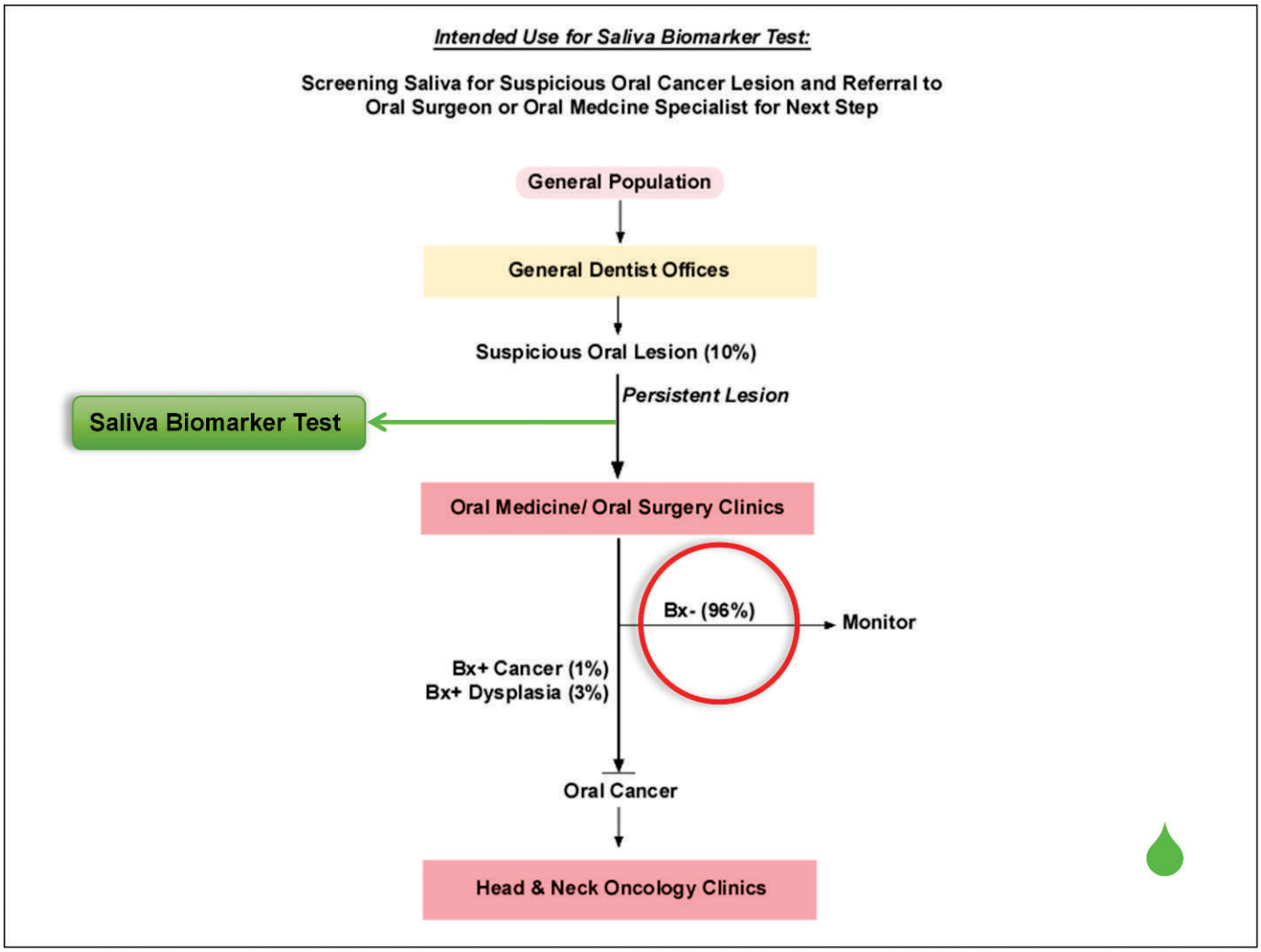

Figure 1. *Intended clinical context of salivary detection of oral cancer in dental office. Salivary diagnostics will reduce the number of unnecessary referrals to hospital for invasive, time-consuming, and expensive diagnostic procedures. Nine-six percent of biopsies of suspicious oral mucosal lesions are not cancer.*

crobial peptides[1] and immunoglobulins.[2] However, the mentioned saliva components only constitute a small part of secreted saliva. Considering the multitude of molecular species found and the unknown reason for their presence in the oral cavity (and subsequent gastrointestinal tract), saliva could play yet undetermined roles in maintaining systemic homeostasis.

Cell-free saliva has been found to contain over a thousand proteins involved in a wide range of biologic functions,[3] as well as messenger RNA (mRNA) and micro RNA (miRNA) transcripts[4-6] and metabolites.[7,8] Saliva proteins, mRNA, miRNA, and metabolites are proving useful for diagnostic purposes, as their increased and decreased occurrence in saliva is being found to reflect oral as well as systemic disease. The complex mixture that forms secreted saliva has called for developments in genomic and proteomic approaches to allow for its analysis and its use as a diagnostic medium.

ADVANCES IN SALIVA GENOMICS, PROTEOMICS, AND STUDY DESIGN

Saliva Analysis Technology

Microarray has allowed for a high throughput analysis of saliva and is the current gold standard for identifying salivary transcripts. Currently, the salivary transcriptome is profiled using microarray technology and validated with quantitative polymerase chain reaction (qPCR). Due to the low concentration of some biomarkers and the small volume of sample that can be collected from some subjects, salivary biomarker analysis has called for innova-

tions in technology to allow for detection of specific low-concentration transcripts.

Work conducted by Hu and others[9] has overcome the limitations of microarray and qPCR by two developments: a universal mRNA-amplification method for the microarray biomarker discovery phase, and in the qPCR validation step, a multiplex preamplification method has been developed and implicated. Thus, the performances of these technologies are not hindered by the low concentration of biomarker transcripts in saliva. Moreover, the multiplex preamplification method can perform simultaneously for many different targets and therefore provides a cost-effective screening with decreased workload as well as allowing for quantitative measurement using a relatively small amount of preamplification product.

Saliva Proteomics Analysis

Disease detection based on salivary protein biomarkers provides its own challenges. Proteins do not generally have long half-lives, although there is much difference from protein to protein. Not only does the nature of peptides cause special requirements for processing and storage of saliva protein analytes, the milieu of the oral cavity also imposes protein degrading factors. Saliva is a complex fluid that contains, among others, proteolytic enzymes that affect the stability of saliva protein analytes over time. Thus, protein-based salivary diagnostics requires immediate processing and/or analysis of saliva samples or the use of freezers and costly protease inhibitors for storage until processing.

In research and laboratory settings, requirements for protein-based detection of disease can be met easily. However, in a clinical setting, protein stabilization without need for freezers and other specialized machinery may be required for the clinical reality of salivary diagnostics. Our laboratory is in the forefront of development of protein stabilization methods that allow for storage of protein-containing saliva samples at room temperature and without expensive reagents and machines. We believe such development will greatly benefit salivary diagnostics and facilitate its implication in clinical practices.

Saliva Biomarker Study Design

The use of salivary biomarkers for detection of breast cancer has been explored since the early 2000s. Such studies[10-14] assessed the use of c-erbB-2, VEGF, EGF, and CEA for saliva biomarker-based breast cancer diagnostics. Thus, these studies looked for known

Figure 2. *The oral fluid nanosensor test of the University of California, Los Angeles: a portable device for point-of-care detection of salivary biomarkers.*

serum biomarkers in saliva. Zhang and others[5] were the first to use *de novo* transcriptomic and proteomic approaches to discovery and validation of a salivary biomarker profile for breast cancer detection.

Prospective-specimen-collection and retrospective-blinded-evaluation (PRoBE) Clinical Design—The use of biomarker for clinical decision-making obviously requires stringent testing of the biomarker's performance. Advances in biotechnology and the biomarker research field have increased dramatically in the last decade. However, the validation of discovered biomarkers has fallen behind.

Guidelines for reporting study results and a phased approach to biomarker development have been around for a while. However, not until recently has a comprehensive guide to biomarker study design been available. A notable advance in salivary biomarker clinical study design is PRoBE (prospective specimen collection and retrospective blinded evaluation, described by Pepe and others[15]). PRoBE design incorporates prospective specimen collection from the target population, collected in a blinded fashion with no knowledge about the patient's outcome. After outcome has been determined, patients with the outcome and control subjects without the outcome are selected randomly, and their specimens are tested in a blinded to case-control status fashion. The incorporation of the PRoBE design in biomarker development (discovery and validation) is critical to ensure the eventual validation of biomarkers in a specific clinical setting.

Salivary Transcriptome

mRNA and miRNA—mRNA and miRNA are transcribed by active cell machinery in response to

normal input in order to perform normal cellular functions and maintain cellular and systemic homeostasis. Transcription of RNA is governed by complex molecular pathways and associated molecules of both intracellular and extracellular origin. mRNA as well as miRNA can be found secreted in association with microvesicular structures in the extracellular milieu of transcribing cells and in biofluids (including blood, urine, and saliva) distant to the transcribing cell.[6,16,17] In the diseased state, transcription of specific mRNAs and miRNAs is altered (suppressed or induced) either in normal immunologic attempts to fight the disease (eg, immune-cell signaling) or by the pathology itself (eg, cancer cell transcripts). Although the exact cellular origin of salivary mRNA is unclear, characterization of mRNA profiles in bodily fluids provides insight into the state of the systems gene transcription network and therefore a reflection of the systems status.

There have been several translational advances in transcriptomic salivary biomarker characterization in the last past few years. Transcriptomic biomarkers for primary Sjögren syndrome have undergone preclinical validation,[18] and mRNA biomarkers for oral squamous cell carcinoma (OSCC) previously discovered in a US cohort[19] have been found to detect disease in a cohort of different ethnicity (Serbian).[20] Zhang and others[5] performed discovery and validation of transcriptomic salivary biomarkers for breast cancer, using a *de novo* approach involving mRNA profiling of matched patients and controls using Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) and then preclinical validation in an independent cohort using quantitative (RT)-qPCR.

Applying PRoBE design, Zhang and others[21] identified salivary mRNA biomarkers for pancreatic cancer with 93.3% sensitivity and 100% specificity in distinguishing patients with early stage resectable pancreatic cancer from healthy patients. Importantly, these biomarkers were found to distinguish pancreatic cancer from chronic pancreatitis with high sensitivity and specificity (both 96.7%). Finally, the ovarian cancer salivary transcriptome profile was discovered in a clinical case control study using HG U133 Plus-2.0 Array (Affymetrix) and then validated with qPCR by Lee and others,[22] achieving 85.7% sensitivity and 91.4% specificity.

miRNA) are short (19–25 nucleotides in length) RNA transcripts that function as posttranscriptional regulators as part of the RNA induced silencing complex of protein synthesis by binding to complementary sequences on the target mRNA transcripts (for an extensive review, see Bartel[23,24]). These novel RNAs have been well-characterized and found to play roles in cell growth, differentiation, apoptosis, pathogen-host interactions, as well as stress responses and immune function and are found in saliva[25] (for extensive review see Lu and others,[25] Zeng,[26] and Stadler and Ruohola-Baker[27]).

miRNAs in several cancer cell types have been demonstrated to be differentially expressed compared with normal cells with expression fold changes in the tens to hundreds. Cancer cell changes in mRNA are comparatively small, and cancer miRNAs also appear to cluster solid tumor-types more truly than mRNA.[25,28,29] These properties make miRNA cancer biomarkers very powerful, and it is likely that miRNA will become of great clinical use in salivary diagnostics. Park and others[6] found two miRNAs (miR-125a and miR-200a) that had significantly reduced levels in the saliva of oral cancer patients compared to controls and found that these two markers could be used for detecting OSCC. Liu and others[30] recently discovered that salivary miRNA-31 (miR-31) was significantly elevated in patients with oral cancer at all stages of disease and tumor size. This study also determined that salivary miR-31 was more abundant than blood miR-31, indicating oral tumor origin of this biomarker.

Salivary Proteome

The proteome is the protein complement of the transcriptome and genome and thus a link between cell biology and genetics. The discovery and study of proteins, their amino acid sequences, and their mRNA precursors have been invaluable to the life sciences. Although mRNA expression profiling may provide a more direct representation of gene-transcription, protein expression profiling provides a more direct representation of cellular function because expression of proteins is not only controlled at the transcription level but also at the level of translation.[31,32] Proteomic analysis of body fluids is therefore an accurate reflection of the life and function, disease and death of cells, organs, and the organism.

The human salivary proteome has been well characterized. Several different classes of salivary protein biomarkers that can assist with diagnosis have been reported. Endothelin-1, a vasoconstrictor, was reported as a potential biomarker for OSCC development in oral lichen planus patients.[33] Interleukin-8 (IL-8), interleukin-1"β (IL-1"β), and glycoprotein M2BP have been reported as salivary

biomarkers for oral cancer,[34-36] and immunoglobulins have long since been described as salivary biomarkers for HIV.[37,38]

Salivary Metabolome

The metabolome is the complement of small-molecule metabolites (metabolic intermediates, signalling molecules, and secondary metabolites) that can be measured in a bio-sample. Just as the transcriptome and proteome, the metabolome changes continually and is a dynamic picture of cellular and organ functions, reflecting gene and protein expression as well as environment. Metabolomic investigations generate quantitative data for many metabolites to elucidate metabolic dynamics related to disease state and drug exposure.[39]

The Salivary Metabolome in Periodontal Disease and Systemic Oncology—Using capillary electrophoresis time-of-flight mass spectrometry, our laboratory identified 57 principal metabolites that can accurately predict the probability of being affected by oral cancer, breast cancer, pancreatic cancer, and periodontal disease. Multiple logistic regression models yielded area under the receiver operating characteristic curves (AUCs) of 0.865 for oral cancer, 0.973 for breast cancer, 0.993 for pancreatic cancer, and 0.969 for periodontal diseases, and cross-validation accuracy AUCs of 0.810, 0.881, 0.994, and 0.954, respectively.[7] Wei and others[8] demonstrated, using ultra-performance liquid chromatography coupled with quadruple time-of-flight mass spectrometry, that a combination of three salivary metabolic biomarkers (valine, lactic acid, and phenylalanine) could discriminate OSCC from controls and oral leukoplakia, with accuracy of 0.89 and 0.97, sensitivity of 86.5% and 94.6%, specificity of 82.4% and 84.4%, and positive predictive values of 81.6% and 87.5%, respectively. These reports clearly demonstrate the utility of salivary metabolomic biomarkers for disease detection and the potential for clinical implementation.

Salivary Microbiome

The human oral microbe identification microarray (HOMIM) is a recent development, where an oligonucleotide- microarray, based on the 16S rRNA, has allowed for the profiling and monitoring of changes in the oral microbiota.[40] Alterations in the bacterial profile of the oral cavity have been found to be associated with several diseases: pancreatic cancer,[41] oral cancer,[42] lung cancer,[43,44] colonic neoplasia and extracolonic malignancy,[45] cardiovascular disease and cerebrovascular disease,[46-48] and preterm birth.[49] These changes are reflections of systemic diseases and therefore offer another salivary diagnostic alphabet.

SALIVARY DIAGNOSTICS

The discovery of salivary biomarkers and their reflection of the system's health status, has naturally led to their exploration as tools for disease detection and diagnostics. Detecting disease at an early stage is imperative to success of therapy in most cancers. In the future, salivary diagnostics will aid in rapid and easily accessible clinical diagnosis, thus potentially allowing for more cases being detected at early stages and thereby decreasing mortality caused by cancers.

Oral Disease Detection

Since the 1990s, salivary diagnostics has been developed for oral disease to monitor periodontal disease and caries risk assessment.[50-52] In 2009, Gursoy and others[53] compared the concentration of a select subset of salivary proteins (elastase, lactate dehydrogenase, IL-1"β, interleukin-6 [IL-6], and tumor necrosis factor-α) and the presence of five pathogens (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, and *Treponema denticola*) in patients with advanced periodontal disease and healthy controls. This study reported an association of salivary IL-1"β and multiple oral pathogens with periodontitis. In a study on dysplastic oral leukoplakia in relation to tobacco habits and periodontitis, Sharma and others[54] found that increasing IL-6 levels correlated with increasing severity of dysplasia, indicating a potential for not only salivary biomarker-based detection of disease, but also determination of disease stage.

Discovery and validation of salivary diagnostic markers for oral cancer detection have been confirmed to be applicable across ethnic backgrounds to discriminate oral cancer from cancer-free subjects.[20] The potential of many different types of salivary biomarkers (metabolomic, transcriptomic [miRNA and mRNA], proteomic, and microbiome) have been described for oral cancer.[6,7,30,33,42]

Systemic Disease Detection

More recently, the advances in biotechnology, salivary diagnostics, genomics, and proteomics have extended the range of salivary diagnostics to systemic disease monitoring. Possibly the most attractive attribute of salivary diagnostics is that of its implica-

tion in systemic disease detection, due to the invasive nature of current clinical practice and in some cases poor accuracy of current standard methods.[5]

Currently, available tests for systemic cancers include a multitude of invasive and/or expensive procedures (magnetic resonance imaging, biopsy, X-ray, computed tomography, exfoliated cytology, positron emission tomography, barium swallow and endoscopy). This, combined with severely decreased prognosis associated with late diagnosis, makes the prospect of salivary diagnostics particularly valuable in oncology.

Pancreatic Cancer

A significant milestone in salivary diagnostics was reached by Zhang and others[21] with the characterization of a salivary transcriptome profile that discriminates patients with early stage resectable pancreatic cancer from cancer-free subjects. The use of the salivary transcriptome for detecting pancreatic cancer was also found to outperform currently used blood-based tests in terms of sensitivity and specificity. Farrell and others[41] further showed that variations in oral microbiota could be used to detect pancreatic cancer as well as pancreatitis.

Lung Cancer

The most frequent cause of cancer-related death in men and the second most common cause in women is lung cancer. Currently, lung cancer may be detected with chest X-ray and computed tomography, and diagnosis is confirmed via biopsy. As such, the current ability to detect lung cancer is limited by stage of disease progression, and more than 75% of cases are diagnosed in the late stages, significantly reducing survival rate. Lung cancer, an often asymptomatic or nonspecific symptom presenting disease, represents one of the greatest needs for salivary biomarkers-based diagnostics today.

Using two-dimensional difference gel electrophoresis and mass spectrometry, Hua and others[55] performed proteomic analysis of saliva of patients with lung cancer. Sixteen candidate lung cancer biomarkers were discovered and further verified in the discovery sample, a prevalidation sample set, and in a lung cancer cell line. Three candidate markers achieved 88.5% sensitivity and 92.3% specificity with an AUC of 0.90.

Breast and Ovarian Cancers

Today, breast cancer detection relies on physical examination and imaging techniques. Emerging technologies, such as molecular analysis of nipple fluid aspirate and ductal lavage,[56,57] may provide improved accuracy and potentially earlier diagnosis but are invasive and therefore limited to high-risk patients. Recently, our laboratory reported the discovery and preclinical validation of transcriptomic and proteomic salivary biomarkers with diagnostic power for breast cancer[5] as well saliva biomarkers for ovarian cancer, the most deadly gynecologic cancer.[22]

Other Systemic Diseases

Not only is salivary diagnostics proving useful in detecting systemic cancers, salivary biomarkers for autoimmune diseases, microbial systemic infections, and diabetes have also been described recently,[2,18,37,58] expanding the potential clinical spectrum of salivary diagnostics.

CONCLUSION: SALIVA DIAGNOSTICS IN THE DENTAL OFFICE FOR CHAIR-SIDE DETECTION OF ORAL AND SYSTEMIC DISEASES

Currently, the decision to use available accurate diagnostic methodology for many diseases is made based on symptoms reported by the patient and the clinical observations made by the physician. As such, the final diagnosis is often negative, thus imposing unnecessary burdens on hospitals and increased waiting time for patients. Salivary diagnostics is intended to provide accessible noninvasive primary testing for diseases and will greatly reduce the burden on hospitals, especially relating to complex, invasive diagnostic procedures yielding negative diagnosis and will significantly reduce the number of "unnecessary" invasive procedures being performed today. These technologies will enable the chair-side detection of oral and systemic disease in the dental office[59] with the potential to advance dentistry into primary healthcare (Figure 1).

Any diagnostic fluid requires proper processing, storage, and transport conditions especially suited to the analytes of interest and the milieu that they are found in to maintain the integrity of the analyte(s). The noninvasive and easily collected nature of saliva will allow for wide use and ultimate ease of access, only if analytes can be cost efficiently and easily stabilized for storage until analysis or transport to laboratory. With the expanding reports of diagnostic-value salivary biomarker profiles, it is timely that technologies for saliva sampling and stabilization as well as point-of-care technology are being developed. Our laboratory has developed methods of stabilizing whole saliva at room temperature without centrifu-

gation by simple addition of a stabilizing reagent as previously reported,[60,61] which will extend the usefulness of salivary diagnostics. A potential of saliva-based diagnostics is noninvasive detection of disease without specially trained professionals by use of point-of-care technology that can allow for sensitive and specific detection of diagnostic biomarkers without any processing of the saliva sample. Developed by our laboratory and supported by the National Institute of Dental and Craniofacial Research, the oral fluid nanosensor test (Figure 2) is a robust portable electrochemical multiplex sensor platform, which is able to detect nucleic acids without amplification of analyte transcripts.[62] Such technology will provide easier access to disease detection in remote and impoverished regions and reduce burdens on health systems worldwide.

Conflict of Interest

The author of this manuscript certifies that he has no proprietary, financial, or other personal interest of any nature or kind in any product, service and/or company that is presented in this article except for the following: Dr Wong reports proprietary interests in RNAmeTRIX Inc, a molecular diagnostic company, and personal interests in intellectual properties in salivary diagnostics technologies.

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REFERENCES

1. Gorr SU (2012) Antimicrobial peptides in periodontal innate defense *Frontiers of Oral Biology* **15** 84-98.
2. Zaka-Ur-Rab Z, Abqari S, Shahab T, Islam N, & Shukla I (2012) Evaluation of salivary anti-Salmonella typhi lipopolysaccharide IgA ELISA for serodiagnosis of typhoid fever in children *Archives of Disease in Childhood* **97(3)** 236-238.
3. Denny P, Hagen FK, Hardt M, Liao L, Yan W, Arellanno M, Bassilian S, Bedi GS, Boontheung P, Cociorva D, Delahunty CM, Denny T, Dunsmore J, Faull KF, Gilligan J, Gonzalez-Begne M, Halgand F, Hall SC, Han X, Henson B, Hewel J, Hu S, Jeffrey S, Jiang J, Loo JA, Ogorzalek Loo RR, Malamud D, Melvin JE, Miroshnychenko O, Navazesh M, Niles R, Park SK, Prakobphol A, Ramachandran P, Richert M, Robinson S, Sondej M, Souda P, Sullivan MA, Takashima J, Than S, Wang J, Whitelegge JP, Witkowska HE, Wolinsky L, Xie Y, Xu T, Yu W, Ytterberg J, Wong DT, Yates JR 3rd, & Fisher SJ (2008) The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions *Journal of Proteome Research* **7(5)** 1994-2006.
4. Li Y, Zhou X, St. John MAR, & Wong DTW (2004) RNA Profiling of cell-free saliva using microarray technology *Journal of Dental Research* **83(3)** 199-203.
5. Zhang L, Xiao H, Karlan S, Zhou H, Gross J, Elashoff D, Akin D, Yan X, Chia D, Karlan B, & Wong DT (2010) Discovery and preclinical validation of salivary transcriptomic and proteomic biomarkers for the non-invasive detection of breast cancer *PLoS ONE* **5(12)** e15573.
6. Park NJ, Zhou H, Elashoff D, Henson BS, Kastratovic DA, Abemayor E, & Wong DT (2009) Salivary microRNA: Discovery, characterization, and clinical utility for oral cancer detection *Clinical Cancer Research* **15(17)** 5473-5477.
7. Sugimoto M, Wong DT, Hirayama A, Soga T, & Tomita M (2010) Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles *Metabolomics: Official Journal of the Metabolomic Society* **6(1)** 78-95.
8. Wei J, Xie G, Zhou Z, Shi P, Qiu Y, Zheng X, Chen T, Su M, Zhao A, & Jia W (2010) Salivary metabolite signatures of oral cancer and leukoplakia *International Journal of Cancer. Journal International du Cancer* Dec 28 [Epub ahead of print].
9. Hu Z, Zimmermann BG, Zhou H, Wang J, Henson BS, Yu W, Elashoff D, Krupp G, & Wong DT (2008) Exon-level expression profiling: A comprehensive transcriptome analysis of oral fluids *Clinical Chemistry* **54(5)** 824-832.
10. Streckfus C, & Bigler L (2005) The use of soluble, salivary c-erbB-2 for the detection and post-operative follow-up of breast cancer in women: The results of a five-year translational research study *Advances in Dental Research* **18(1)** 17-24.
11. Streckfus C, Bigler L, Dellinger T, Dai X, Kingman A, & Thigpen JT (2000) The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: A preliminary study *Clinical Cancer Research* **6(6)** 2363-2370.
12. Navarro MA, Mesia R, Diez-Gibert O, Rueda A, Ojeda B, & Alonso MC (1997) Epidermal growth factor in plasma and saliva of patients with active breast cancer and breast cancer patients in follow-up compared with healthy women *Breast Cancer Research and Treatment* **42(1)** 83-86.
13. Brooks MN, Wang J, Li Y, Zhang R, Elashoff D, & Wong DT (2008) Salivary protein factors are elevated in breast cancer patients *Molecular Medicine Reports* **1(3)** 375-378.
14. Bigler LR, Streckfus CF, Copeland L, Burns R, Dai X, Kuhn M, Martin P, & Bigler SA (2002) The potential use of saliva to detect recurrence of disease in women with breast carcinoma *Journal of Oral Pathology & Medicine* **31(7)** 421-431.
15. Pepe MS, Feng Z, Janes H, Bossuyt PM, & Potter JD (2008) Pivotal evaluation of the accuracy of a biomarker used for classification or prediction: Standards for study design *Journal of the National Cancer Institute* **100(20)** 1432-1438.
16. Garcia JM, Garcia V, Peña C, Domínguez G, Silva J, Diaz R, Espinosa P, Citores MJ, Collado M, & Bonilla F (2008) Extracellular plasma RNA from colon cancer patients is confined in a vesicle-like structure and is mRNA-enriched *RNA* **14(7)** 1424-1432.
17. Tinzl M, Marberger M, Horvath S, & Chypre C (2004) DD3PCA3 RNA analysis in urine—A new perspective for detecting prostate cancer *European Urology* **46(2)** 182-186; discussion 87.

18. Hu S, Gao K, Pollard R, Arellano-Garcia M, Zhou H, Zhang L, Elashoff D, Kallenberg CG, Vissink A, & Wong DT (2010) Preclinical validation of salivary biomarkers for primary Sjögren's syndrome *Arthritis Care & Research* **62(11)** 1633-1638.

19. Li Y (2004) Salivary transcriptome diagnostics for oral cancer detection *Clinical Cancer Research* **10(24)** 8442-8450.

20. Brinkmann O, Kastratovic DA, Dimitrijevic MV, Konstantinovic VS, Jelovac DB, Antic J, Nesic VS, Markovic SZ, Martinovic ZR, Akin D, Spielmann N, Zhou H, & Wong DT (2011) Oral squamous cell carcinoma detection by salivary biomarkers in a Serbian population *Oral Oncology* **47(1)** 51-55.

21. Zhang L, Farrell JJ, Zhou H, Elashoff D, Akin D, Park NH, Chia D, & Wong DT (2010) Salivary transcriptomic biomarkers for detection of resectable pancreatic cancer *Gastroenterology* **138(3)** 949-957.e7.

22. Lee Y-H, Kim JH, Zhou H, Kim BW, & Wong DT (2012) Salivary transcriptomic biomarkers for detection of ovarian cancer: For serous papillary adenocarcinoma *Journal of Molecular Medicine* **90(4)** 427-434.

23. Bartel DP (2009) MicroRNAs: Target recognition and regulatory functions *Cell* **136(2)** 215-233.

24. Bartel DP (2004) MicroRNAs: Genomics, biogenesis, mechanism, and function *Cell* **116(2)** 281-297.

25. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, & Golub TR (2005) MicroRNA expression profiles classify human cancers *Nature* **435(7043)** 834-838.

26. Zeng Y (2006) Principles of micro-RNA production and maturation *Oncogene* **25(46)** 6156-6162.

27. Stadler BM, & Ruohola-Baker H (2008) Small RNAs: Keeping stem cells in line *Cell* **132(4)** 563-566.

28. Mi S, Lu J, Sun M, Li Z, Zhang H, Neilly MB, Wang Y, Qian Z, Jin J, Zhang Y, Bohlander SK, Le Beau MM, Larson RA, Golub TR, Rowley JD, & Chen J (2007) MicroRNA expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia *Proceedings of the National Academy of Sciences of the United States of America* **104(50)** 19971-19976.

29. Jiang J, Lee EJ, Gusev Y, & Schmittgen TD (2005) Real-time expression profiling of microRNA precursors in human cancer cell lines *Nucleic Acids Research* **33(17)** 5394-5403.

30. Liu CJ, Lin SC, Yang CC, Cheng HW, & Chang KW (2012) Exploiting salivary miR-31 as a clinical biomarker of oral squamous cell carcinoma *Head & Neck* **34(2)** 219-224.

31. Wilkie GS, Dickson KS, & Gray NK (2003) Regulation of mRNA translation by 5′- and 3′-UTR-binding factors *Trends in Biochemical Sciences* **28(4)** 182-188.

32. Padmanabhan K, & Richter JD (2006) Regulated Pumilio-2 binding controls RINGO/Spy mRNA translation and CPEB activation *Genes & Development* **20(2)** 199-209.

33. Cheng YS, Rees T, Jordan L, Oxford L, O'Brien J, Chen HS, & Wong D (2011) Salivary endothelin-1 potential for detecting oral cancer in patients with oral lichen planus or oral cancer in remission *Oral Oncology* **47(12)** 1122-1126.

34. St John MA, Li Y, Zhou X, Denny P, Ho CM, Montemagno C, Shi W, Qi F, Wu B, Sinha U, Jordan R, Wolinsky L, Park NH, Liu H, Abemayor E, & Wong DT (2004) Interleukin 6 and interleukin 8 as potential biomarkers for oral cavity and oropharyngeal squamous cell carcinoma *Archives of Otolaryngology–Head & Neck Surgery* **130(8)** 929-935.

35. Wei F, Patel P, Liao W, Chaudhry K, Zhang L, Arellano-Garcia M, Hu S, Elashoff D, Zhou H, Shukla S, Shah F, Ho CM, & Wong DT (2009) Electrochemical sensor for multiplex biomarkers detection *Clinical Cancer Research* **15(13)** 4446-4452.

36. Elashoff D, Zhou H, Reiss JK, Wang J, Xiao H, Henson B, Hu S, Arellano M, Sinha U, Le A, Messadi D, Wang M, Nabili V, Lingen M, Morris D, Randolph T, Feng Z, Akin D, Kastratovic DA, Chia D, Abemayor E, & Wong DT (2012) Pre-validation of salivary biomarkers for oral cancer detection *Cancer Epidemiology, Biomarkers and Prevention* **21(4)** 664-667.

37. Emmons W (1997) Accuracy of oral specimen testing for human immunodeficiency virus *American Journal of Medicine* **102(4A)** 15-20.

38. Malamud D (1997) Oral diagnostic testing for detecting human immunodeficiency virus-1 antibodies: A technology whose time has come *American Journal of Medicine* **102(4A)** 9-14.

39. Spielmann N, & Wong DT (2011) Saliva: Diagnostics and therapeutic perspectives *Oral Diseases* **17(4)** 345-354.

40. Huyghe A, Francois P, Charbonnier Y, Tangomo-Bento M, Bonetti EJ, Paster BJ, Bolivar I, Baratti-Mayer D, Pittet D, & Schrenzel J; Geneva Study Group on Noma (GESNOMA) (2008) Novel microarray design strategy to study complex bacterial communities *Applied and Environmental Microbiology* **74(6)** 1876-1885.

41. Farrell JJ, Zhang L, Zhou H, Chia D, Elashoff D, Akin D, Paster BJ, Joshipura K, & Wong DT (2012) Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer *Gut* **61(4)** 582-588.

42. Mager DL, Haffajee AD, Devlin PM, Norris CM, Posner MR, & Goodson JM (2005) The salivary microbiota as a diagnostic indicator of oral cancer: A descriptive, non-randomized study of cancer-free and oral squamous cell carcinoma subjects *Journal of Translational Medicine* **3** 27.

43. Anttila T, Koskela P, Leinonen M, Laukkanen P, Hakulinen T, Lehtinen M, Pukkala E, Paavonen J, & Saikku P (2003) *Chlamydia pneumoniae* infection and the risk of female early-onset lung cancer *International Journal of Cancer. Journal International du Cancer* **107(4)** 681-682.

44. Koyi H, Branden E, Gnarpe J, Gnarpe H, & Steen B (2001) An association between chronic infection with *Chlamydia pneumoniae* and lung cancer. A prospective 2-year study *APMIS: Acta Pathologica, Microbiologica, et Immunologica Scandinavica* **109(9)** 572-580.

45. Gold JS, Bayar S, & Salem RR (2004) Association of *Streptococcus bovis* bacteremia with colonic neoplasia and extracolonic malignancy *Archives of Surgery* **139(7)** 760-765.

46. Joshipura KJ, Douglass CW, & Willett WC (1998) Possible explanations for the tooth loss and cardiovascular disease relationship *Annals of Periodontology / the American Academy of Periodontology* **3(1)** 175-183.

47. Meurman JH, Sanz M, & Janket SJ (2004) Oral health, atherosclerosis, and cardiovascular disease *Critical Reviews in Oral Biology and Medicine* **15(6)** 403-413.

48. Morrison HI, Ellison LF, & Taylor GW (1999) Periodontal disease and risk of fatal coronary heart and cerebrovascular diseases *Journal of Cardiovascular Risk* **6(1)** 7-11.

49. Goldenberg RL, & Culhane JF (2006) Preterm birth and periodontal disease *New England Journal of Medicine* **355(18)** 1925-1927.

50. Kornman KS, Crane A, Wang HY, di Giovine FS, Newman MG, Pirk FW, Wilson TG Jr, Higginbottom FL, & Duff GW (1997) The interleukin-1 genotype as a severity factor in adult periodontal disease *Journal of Clinical Periodontology* **24(1)** 72-77.

51. Socransky SS, Haffajee AD, Smith C, & Duff GW (2000) Microbiological parameters associated with IL-1 gene polymorphisms in periodontitis patients *Journal of Clinical Periodontology* **27(11)** 810-818.

52. Baughan LW, Robertello FJ, Sarrett DC, Denny PA, & Denny PC (2000) Salivary mucin as related to oral *Streptococcus mutans* in elderly people *Oral Microbiology and Immunology* **15(1)** 10-14.

53. Gursoy UK, Könönen E, Uitto VJ, Pussinen PJ, Hyvärinen K, Suominen-Taipale L, & Knuuttila M (2009) Salivary interleukin-1beta concentration and the presence of multiple pathogens in periodontitis *Journal of Clinical Periodontology* **36(11)** 922-927.

54. Sharma M, Bairy I, Pai K, Satyamoorthy K, Prasad S, Berkovitz B, & Radhakrishnan R (2011) Salivary IL-6 levels in oral leukoplakia with dysplasia and its clinical relevance to tobacco habits and periodontitis *Clinical Oral Investigations* **15(5)** 705-714.

55. Hua X, Zhang L, Zhou H, Lee JM, Garon EB, & Wong DT (2012) Proteomic analysis of human saliva from lung cancer patients using two-dimentional difference gel elecrtophoresis and mass spectometry *Molecular and Cell Proteomics: MCP* **11(2)** M111.012112.

56. Sauter ER (2005) Analysis of nipple aspirate fluid for diagnosis of breast cancer: An alternative to invasive biopsy *Expert Review of Molecular Diagnostics* **5(6)** 873-881.

57. Li J, Zhao J, Yu X, Lange J, Kuerer H, Krishnamurthy S, Schilling E, Khan SA, Sukumar S, & Chan DW (2005) Identification of biomarkers for breast cancer in nipple aspiration and ductal lavage fluid *Clinical Cancer Research* **11(23)** 8312-8320.

58. Rao PV, Reddy AP, Lu X, Dasari S, Krishnaprasad A, Biggs E, Roberts CT, & Nagalla SR (2009) Proteomic identification of salivary biomarkers of type-2 diabetes *Journal of Proteome Research* **8(1)** 239-245.

59. Greenberg BL, Glick M, Frantsve-Hawley J, & Kantor ML (2010) Dentists' attitudes toward chairside screening for medical conditions *Journal of the American Dental Association* **141(1)** 52-62.

60. Jiang J, Park NJ, Hu S, & Wong DT (2009) A universal pre-analytic solution for concurrent stabilization of salivary proteins, RNA and DNA at ambient temperature *Archives of Oral Biology* **54(3)** 268-273.

61. Park NJ, Yu T, Nabili V, Brinkman BM, Henry S, Wang J, & Wong DT (2006) RNAprotect saliva: An optimal room-temperature stabilization reagent for the salivary transcriptome *Clinical Chemistry* **52(12)** 2303-2304.

62. Gau V, & Wong D (2007) Oral fluid nanosensor test (OFNASET) with advanced electrochemical-based molecular analysis platform *Annals of the New York Academy of Sciences* **1098** 401-410.