Salivary Diagnostics: Moving to the Next Level

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Upon successful completion of this CE activity 2 CE credit hours may be awarded.
LEARNING OBJECTIVES
After participating in this CE activity, the individual will learn:
• The main principles of salivary biomarker research.
• The importance of early detection of oral cancer especially regarding the current shift in risk profile.

ABOUT THE AUTHORS

Dr. Brinkmann finished his DMD and PhD program at Charité, University Medicine Berlin, Germany. After working as a dentist in private practice, he joined Dr. David T. W. Wong’s lab at University of California at Los Angeles’ Dental Research Institute as a postdoctoral scholar to work on salivary diagnostics for oral cancer detection. He can be reached via e-mail at ole.brinkmann@gmail.com.

Disclosure: Dr. Brinkmann is a clinical research manager for DENTSPLY. He reports no conflict of interest, as he is not currently working in the field of salivary diagnostics.

Dr. Spielmann pursued her PhD in the department of Sports Medicine at the Humboldt University of Berlin, Germany. Subsequently she started her postdoctoral training in the Human Genomics Laboratory at the Pennington Biomedical Research Center in Baton Rouge, La, and at the Pierre and Marie Curie University, INSERM, department of nutrition, Paris, France. Dr. Spielmann’s research in Dr. David T. W. Wong’s laboratory at the University of California at Los Angeles’ Dental Research Institute is focused on profiling the salivary transcriptome via deep sequencing and investigating xenograft mouse models for basic salivary research. She can be reached via e-mail at spielmann.nadine@gmail.com.

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SALIVA—MORE THAN WATER
“Saliva doesn’t have the drama of blood, it doesn’t have the integrity of sweat, it doesn’t have the emotional appeal of tears,” stated Dr. Irwin Mandel,1 professor emeritus at the Columbia University College of Dental Medicine (New York City).

Most of the time, saliva only attracts people’s attention when it is absent. The center of interest for diagnostic purposes has always been blood, while saliva was at best sidelined. One of the primary reasons for this lack of appreciation might be the fact that saliva contains 99% water. However, the remaining one percent is harboring a real treasure due to an astonishing complexity of genomic, proteomic, transcriptomic, metabolomic, and microbiomic molecules.

“If saliva were water, we would have little stumps of teeth or no teeth at all by age 20 years—we would have dissolved our teeth away,” stated Dr. Frank Oppenheim,1 professor and chairman of the Department of Periodontology and Oral Biology at Boston University.

Not only is saliva a direct filtrate of blood, it is also accessible in a completely noninvasive, easy, safe, and low-cost manner. Using saliva as a diagnostic medium could have some of the most impactful medical applications; taking a saliva sample is so easy, one does not need trained personnel to accomplish it—patients can basically collect their own samples in an unsupervised manner.
Imagine taking salivary diagnostics to the next level, using saliva as a powerful fluid for early detection and the first line of diagnosis for life-threatening diseases such as cancer, metabolic disorders, infections, and inflammatory diseases. As salivary glands and the oral cavity truly represent a dentist’s field of expertise, this is a unique moment where the dentist’s world may be paving a new path for general medicine.

This article discusses the evolution of salivary diagnostics, current research, and future directions, with an emphasis on advancements in the diagnosis of oral cancer using saliva as a diagnostic fluid.

THE NEED FOR BIOMARKERS
The first steps in the direction of salivary diagnostics were already taken decades ago with the discovery of how salivary hypothiocyanide can be used to discriminate smokers from nonsmokers. But for the longest time, the only relevant saliva test was based on the abundance of *Streptococcus mutans* and *Streptococcus lactobacillae* in combination with salivary flow rate and buffering capacity, with limited predictive value for estimating caries risk.

In order to use a fluid as a diagnostic medium, biomarkers are needed. Biomarkers describe measurable reproducible biological parameters used to gather information about health conditions. Any kind of a molecule—protein, RNA, metabolite, DNA, microflora—has the potential to serve as a biomarker for any state of disease. Carriers for these molecules can be every human tissue or fluid. The question is not whether a certain biomarker exists, but rather: are we smart enough to detect and identify the most discriminatory biomarkers in saliva?

THE IMPACT OF ORAL CANCER
As a recent proof of principle for salivary diagnostics, the first disease of major impact was oral cancer. Often falsely underestimated as an “orphan” disease, oral cancer is the sixth most common cancer worldwide and is diagnosed 400,000 times each year; 35,000 of these cases occur in the United States, killing 8,000 annually, with males outscoring females and a 5-year survival rate remaining low for decades at an unacceptable 60%.

In developed countries, the main risk factors for oral cancer remain alcohol and tobacco abuse, especially in combination. For the longest time, the typical oral cancer patient was the heavy-smoking, heavy-drinking high-age male.

This paradigm is changing quickly as we are currently observing a dramatic shift, with fast rising incidences in human papillomavirus (HPV)-associated oropharyngeal cancer in nonsmoking nondrinking middle-aged populations. For this form of cancer, the subtype HPV16 presents the main risk factor, with HPV16 infection strongly correlating with sexual behavior. As a general indicator for this rising prevalence, the rate of HPV16 positive tonsil tumors in Sweden has risen from 28% to 68% during the last 30 years.

The key for survival in any case of cancer is early detection. Diagnosed at early stages, the 5-year survival rate for oral cancer patients ranges from 71% to 96% depending on tumor location, while at late and progressed stages it drops to only 30% to 48%. Sixty percent to 71% of all oral cancer cases are only diagnosed at Stage III or IV, which presents the main reason for the high fatality rate. Oral cancer is one of the most expensive cancers to treat, and the healthcare expenses for therapy increase exponentially as the tumor progresses, while the patient's life quality after treatment drops dramatically. Not only would early detection significantly improve survival and life quality for patients; it would also release economic resources that could be used for other urgent healthcare purposes.

The often late diagnosis of oral cancer seems surprising, since the oral cavity is easily accessible for visual inspection and, unlike most other parts of the body, is examined by dentists on a more or less regular basis. There are 2 main reasons that hinder the success of visual oral inspection: nonaccess to high-risk populations, and nonawareness by dental professionals.

In the first case, the “classic” heavy-smoking heavy-drinking male oral cancer patient, who still outscores the new HPV-related patients, is likely to be less health aware in general compared to a normal nonsmoking, nondrinking population, and thus less likely to consult any kind of medical facility on a regular basis. And secondly, dentists tend to neglect performing a full oral cavity examination and rather concentrate on their comfort zone—the teeth. This observation is supported by an ADA survey which found
that only 15% of patients reported receiving a thorough oral cavity examination by their dentists.

In this context, it is also important to mention that failure to diagnose oral cancer is the number 2 reason for malpractice claims against American dentists, settling for an average of one million dollars and turning the economic impact of oral cancer quickly into a serious liability issue for dentists. 12

SALIVARY DIAGNOSTIC TOOLS

To test the discriminatory power of salivary biomarkers as a potential alternative to regular visual inspection, saliva samples were taken from US cohorts of still untreated oral squamous cell carcinoma (OSCC) patients and healthy controls. 13 As the biggest subgroup, OSCC represents 90% of all oral cancers. 6 The samples were processed according to established protocols and tested for messenger ribonucleic acid (mRNA) and protein markers. 13, 14 After microarray identification of mRNA candidates, 7 salivary markers were verified with quantitative real-time (qRT)-polymerase chain reaction (PCR) (interleukin-1 beta [IL-1ß]; IL-8; dual specificity protein phosphatase 1; ornithine decarboxylase antizyme 1; H3F3A; spermidine/spermine N1-acetyltransferase 1; S100-protein) showing a combined 91% sensitivity and 91% specificity. 13 Salivary proteins contributed 7 additional markers (IL-8, IL-1ß, mac-2-binding protein, myeloid-related protein 14, CD59, catalase, profilin) with 90% sensitivity and 83% specificity, most of them identified with 2-dimensional gel electrophoresis followed by mass spectrometry and immunoassay verification. 14, 16 These findings were independently validated in OSCC cohorts from India and Serbia. 17, 18 In addition, 2 studies also detected highly discriminatory microRNAs (miRNAs) and metabolites in saliva of OSCC patients 19, 20 and strengthened the salivary OSCC findings.

Only a few years ago, it was found that IL-8 protein is accompanied by the presence of IL-8 mRNA. 16 This came as a surprise, since saliva was classically perceived as too hostile a medium for such a sensitive molecule as RNA. While in the beginning, the presence of salivary RNA was discussed controversially, salivary RNA diagnostics quickly became a widely accepted and highly dynamic field. At the moment, salivary RNA has turned out to be the most promising and fruitful biomarker source. Although salivary mRNA in comparison to tissue RNA in general is fragmented and partially degraded, the mRNA signal is highly robust and reproducible. 21 Currently, researchers have access to high throughput microarray platforms, tools that enable performing parallel screening of thousands of mRNA sequences in one sample while consecutive qPCR allows highly sensitive verification of individual signals. This approach has allowed researchers to put salivary mRNA biomarker detection in context to virtually any clinical condition.

Our laboratory is processing a number of high-impact diseases using expression-based microarray analysis and qRT-PCR. Highly discriminatory salivary transcriptomic biomarkers have been identified for Sjögren’s syndrome, pancreatic cancer, ovarian cancer, and breast cancer. 22-26 Furthermore, xenograft mouse models are being used to provide functional data aiming for a systemic proof of concept for salivary biomarkers showing that human tumor markers are trafficking from the tumor site into saliva.

The successful detection of disease-specific biomarkers in oral and nonoral diseases is a milestone in the history of saliva diagnostics, and still there is one central question that needs to be addressed: Where do these biomarkers come from and how do they enter the oral cavity?

The source of salivary mRNA as well as its biological function still remain unclear. Step by step, the puzzle is being unraveled. The salivary glands as well as the oral epithelium and the gingival crevice fluid contribute to the whole salivary mRNA pool. It has been successfully shown in a xenograft mouse model that induction of melanoma leads to distinct salivary microarray expression profile changes. 27 In another study, it has been demonstrated that salivary RNA through association with yet unidentified macromolecules is surprisingly stable even at room temperature, but rapidly degrades when Triton X, a surface surfactant, is added. 28 These findings merged into the current hypothesis that exosomes—lipid layer coated microvesicles—are the most important salivary mRNA protective escorts traveling from disease sites into the oral cavity. Isolated salivary exosomes have been shown to contain intact functional mRNA that can even change oral keratinocyte protein expression patterns, and thus can act as a “cargo shuttle” in this process. 29 These findings are opening up a completely new direction of functional salivary research.
FROM LAB BENCH TO CHAIRSIDE

It is simple to take a saliva sample, but the steps following sampling are still not convenient enough for widespread clinical application. The major technical roadblock for salivary diagnostics has been presented by the fact that all saliva samples had to be kept on ice followed by a low-speed centrifugation (2,600 g, 4°C, 15 minutes). After addition of RNA and protein stabilizers, the samples had to be kept at negative 80°C until analysis. This method is well suited for research purposes, but obviously not for saliva testing in dental offices or low- to medium-equipped medical facilities. For that reason, customizing saliva sampling to be more convenient and less sensitive to ambient conditions is necessary. As a first breakthrough, we have been able to show that salivary mRNA is stable at room temperature for at least 10 weeks without any stabilizers.\(^{30}\)

The final goal is to turn salivary diagnostics into a truly impactful application. Substantial resources are currently put into developing the technical means for a point-of-care salivary diagnostic platform, so far with a more than encouraging outcome. The oral fluid nanosensor test device (OFNASET) presents a chairside, handheld device detecting salivary constituents within minutes, with the potential of providing a highly sensitive medical diagnostic infrastructure to the general public or even the most deprived regions in the world. Among the large number of microsensor prototypes that are developed by companies and research groups around the globe, the OFNASET is the first platform that allows parallel multiple detection of RNA and protein markers. It has been optimized for salivary biomarker detection and has recently proven its value in a cohort of Indian oral cancer patients.\(^{18}\) Further engineering advances have led to the development of a next generation salivary diagnostic platform: the novel saliva-based, point-of-care technology (POCT) (Figure 1).

The last remaining step necessary for translation of oral cancer diagnostics from the academic lab into a real world clinical application is the large-scale multi-institutional validation of the oral cancer markers. In accordance with the guidelines of the Early Research Detection Network, this definitive validation will be conducted in a prospective specimen collection retrospective blinded evaluation (PRoBE) design manner, ensuring unbiased and most clinically relevant biomarker performance.\(^{31}\) A PRoBE design validation presents a tall task and is often the bottleneck for research in the biomarker field. With only a few disease biomarkers reaching sensitivity and specificity levels in the range of oral cancer salivary biomarkers, we are most excited and eager to encounter this final step.

SOMEDAY SOON

The saliva diagnostics group at the University of California at Los Angeles School of Dentistry is following the vision of using saliva for disease diagnostics as well as for normal health surveillance. They are eager to achieve the goal that someday soon dentists will be able to collect saliva specimens and use the chairside POCT to screen patients for any number of conditions and diseases. As author Dr. David T.W. Wong states, ‘The goal is to turn this biofluid into a resource that can be used in doctors’ and dentists’ offices
for a large number of clinical applications and early detection of diseases.”

Recently, the salivaomics knowledge base (SKB) (Figure 2), a Web-based data management system dedicated to help clinicians use saliva as a diagnostic tool, has been created.32 The SKB is a data repository, a data management system, and a Web resource designed to support human salivary proteomics, transcriptomics, miRNA, metabolomics, and microbiome research. The SKB will provide the first Web resource dedicated to salivaryomics studies. It will contain the data and information needed to explore the biology, diagnostic potentials, pharmacoproteomics, and pharmacogenomics of human saliva (available at skb.ucla.edu). Overall, the SKB will allow a systems approach to the utilization of salivary diagnostic technology for personalized medical applications.

**SUMMARY**

The evolution of salivary diagnostics has reached a new level toward the goal of using saliva as a powerful fluid for early detection and the first line of diagnosis for life-threatening diseases such as cancer, metabolic disorders, infections, and inflammatory diseases. Newly developed tools such as the novel saliva-based POCT and the SKB are helping to realize the goal of making salivary diagnostics available to clinicians worldwide. This is a unique moment where dentistry may be paving a new path for primary healthcare.

![Salivaomics Knowledge Base](Figure 2)
Salivary Diagnostics: Moving to the Next Level

REFERENCES


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POST EXAMINATION QUESTIONS

1. What is (are) the classic risk factor(s) for developing oral cancer in the United States?
   a. Exposure to arsenic.
   b. Alcohol and tobacco.
   c. Sexual behavior.
   d. Bisphosphonates.

2. What is the yearly incidence for oral cancer in the United States?
   a. 5,000.
   b. 15,000.
   c. 25,000.
   d. 35,000.

3. How many people die of oral cancer each year in the United States?
   a. 5,000.
   b. 8,000.
   c. 11,000.
   d. 14,000.

4. What is the 5-year survival rate for oral cancer patients?
   a. 90%.
   b. 80%.
   c. 70%.
   d. 60%.

5. Which salivary parameter can be used to discriminate smokers from nonsmokers?
   a. Hypothiocyanide level.
   b. Salivary buffer capacity.
   c. Salivary flow rate.
   d. mRNA level.

6. Which detection method is used for verification of salivary messenger RNA biomarker candidates?
   a. Mass spectrometry.
   b. Immunohistochemistry.
   c. Quantitative real time polymerase chain reaction.
   d. Microarray.

7. Which autoimmune disease has been detectable in saliva?
   a. Lupus erythematosus.
   b. Rheumatoid arthritis.
   c. Sjögren's syndrome.
   d. Crohn's disease.
8. Saliva is comprised of ____ water.
   a. 88%.
   b. 93%.
   c. 97%.
   d. 99%.

9. What percentage of oral cancers is classified as oral squamous cell cancers?
   a. 90%.
   b. 80%.
   c. 70%.
   d. 60%.

10. According to current hypothesis, what is the protective mechanism against RNA degradation in saliva?
    a. Intracellular transport.
    b. Intraexosomal transport.
    c. Association with glycoproteins.
    d. Active secretion of RNAse inhibitors.

11. What is the unique novelty of the oral fluid nanosensor test device?
    a. Parallel detection of RNA and protein markers.
    b. Usable for any kind of body fluid.
    c. Is a further development of current brush biopsy.
    d. First biomarker microsensor platform.

12. Which infection is associated with the current increase in oropharyngeal cancer incidence?
    a. HIV.
    b. Hepatitis C.
    c. Human papillomavirus 16.
    d. Epstein-Barr virus.

13. Which parameter(s) describe(s) the performance of biomarkers?
    a. Sensitivity and specificity.
    b. Prevalence and incidence.
    c. Survival rate.
    d. EC50.

14. How many mRNA markers have been identified for the detection of oral cancer?
    a. 5.
    b. 6.
    c. 7.
    d. 8.

15. What is one of the main diagnostic advantages of saliva compared to other body fluids or tissues?
    a. Noninvasive access.
    b. Access to high volumes (1.5 liters per day).
    c. Saliva contains 99% water, providing highly pure biomarker molecules.
    d. Salivary glycoprotein matrix prevents biomarker degradation.

16. What is one of the main characteristics of salivary mRNA?
    a. Fragmented.
    b. Highly concentrated.
    c. Only few RNA sequences.
    d. Nonhuman origin.
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