Published in final edited form as:

Clin Ther. 2015 March 1; 37(3): 529–539. doi:10.1016/j.clinthera.2015.02.022.

Not All Biofluids Are Created Equal: Chewing Over Salivary Diagnostics and the Epigenome

M.E. Wren^{1,2}, E.A. Shirtcliff³, and Stacy S. Drury^{1,2}

¹Neuroscience Graduate Program, Tulane University, New Orleans, Louisiana

²Department of Psychiatry and Behavioral Health Science, Tulane University School of Medicine, New Orleans, Louisiana

³Department of Human Development and Family Studies, Iowa State University, Ames, Iowa

Abstract

Purpose—This article describes progress to date in the characterization of the salivary epigenome and considers the importance of previous work in the salivary microbiome, proteome, endocrine analytes, genome, and transcriptome.

Methods—PubMed and Web of Science were used to extensively search the existing literature (original research and reviews) related to salivary diagnostics and bio-marker development, of which 125 studies were examined. This article was derived from the most relevant 73 sources highlighting the recent state of the evolving field of salivary epigenomics and contributing significantly to the foundational work in saliva-based research.

Findings—Validation of any new saliva-based diagnostic or analyte will require comparison to previously accepted standards established in blood. Careful attention to the collection, processing, and analysis of salivary analytes is critical for the development and implementation of newer applications that include genomic, transcriptomic, and epigenomic markers. All these factors must be integrated into initial study design.

Implications—This commentary highlights the appeal of the salivary epigenome for translational applications and its utility in future studies of development and the interface among environment, disease, and health.

Keywords

biomarker; DNA methylation; epigenome; salivary diagnostics; salivary transcriptome; telomere length

© 2015 Published by Elsevier HS Journals, Inc.

CONFLICTS OF INTEREST

This study was supported by grant R01 MH101533-01 from the National Institute of Mental Health to Dr Stacy S. Drury, principal investigator. The project described was also supported by award K12HD043451 (to Dr Stacy S. Drury) from the Eunice Kennedy Shriver National Institute of Child Health and Human Development. The content is solely the responsibility of the authors and does not necessarily represent the official views of the Eunice Kennedy Shriver National Institute of Child Health & Human Development or the National Institutes of Health. The authors have indicated that they have no other conflicts of interest regarding the content of this article.

INTRODUCTION TO SALIVARY ANALYTICS

The range of biomarker applications for human saliva in basic research and clinical diagnostics has flourished recently. Contrary to other human biofluids, saliva is accessible noninvasively, available on demand, and rapidly renewable in most healthy adults, adolescents, and children. Saliva is relatively inexpensive and quick to collect. Salivary sampling and analysis from individual patients or larger study populations are adaptable to most fields of medical science—from pathophysiology and developmental genetics to microbiology and neuropsychiatry.^{1–3} Valid characterization of saliva samples, including the salivary proteome (ie, total protein), transcriptome (ie, total RNA), and epigenome (eg, methylation and telomere length), is measurable now through a range of powerful biochemical techniques, such as flow cytometry, mass spectrometry, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), microarray chips, and deep sequencing.^{4–6} Adaptation of these biomolecular assays for saliva represents a significant leap forward in saliva-based analytics and further establishes the foundation for translational salivary diagnostics.

This article presents an overview of the current considerations and controversies relevant to salivary diagnostics and whether the field has sufficiently overcome these issues to successfully measure epigenomic markers. Issues of sample collection and specific analytics will be discussed within the framework of a critical comparison between blood and saliva. The end goal of this discussion is to ignite interest in the pursuit of new salivary biomarkers while addressing the appropriate selection of collection and measurement strategies needed to advance translational studies of human health and development.

PubMed and Web of Science were used to extensively search the existing literature (original research and reviews) related to salivary diagnostics and bio-marker development, of which 125 studies were examined. This article was derived from the most relevant 73 sources highlighting the recent state of the evolving field of salivary epigenomics and contributing significantly to the foundational work in saliva-based research.

Oral Diagnostics with Saliva

The foundation of modern salivary research began with oral health clinicians seeking to identify and characterize local markers of oral disease and salivary gland disorder.⁷ The discovery and validation of salivary analytes associated with human health and development encouraged broadening beyond this early focus on individual oral pathology to include large-scale, longitudinal clinical research and translational applications.⁸ In 2002, the National Institute of Dental and Craniofacial Research funded a program entitled “Development and Validation Technologies for Saliva Based Diagnostics” to support interdisciplinary research on practical salivary biomarkers for the diagnosis of systemic disease. The program has supported studies advancing salivary diagnostics of pulmonary, cardiac, and infectious diseases, as well as cancer biomarker research.⁹ Much of this effort is directly or indirectly illustrated by the research presented throughout this special issue, indicating that the development and application of diverse salivary analytics are now firmly established.

Systemic Diagnostics with Saliva

The objective of salivary biomarker development is evolving toward an inclusive and expansive assessment of systemic pathology. This article highlights genetically informative analytes because these are new health-related approaches capable of bridging a significant gap in clinical methods from oral pathology to overall health and development. Throughout, a consistent theme emerges in which major advantages of salivary diagnostics over blood-dependent methods (eg, collection, processing, and assaying) has allowed for a new set of saliva-specific biomarkers to emerge.

Advancement in the practicality, efficiency, and sensitivity of biomolecular assay-based techniques resulted in the transition from specific bioindicators of oral health in saliva to systemic biomarkers with comprehensive utility across health outcomes. Addressing developmental and epigenetic regulation of physiologic processes is a logical next step in salivary analytics because these biomarkers lie at the intersection between genetic inheritance and environment-dependent adaptation. Accomplishing this goal requires the identification of specific and reliable salivary epigenetic markers reflective of environmentally responsive physiologic changes capable of affecting the relationship between life-course exposures and maladaptive health outcomes across development and aging.^{10–13}

CHOOSING SALIVA: CONTEXTUAL ASPECTS

To appreciate the utility of salivary analytics for research and translational applications, it is important to first consider the functional association of saliva and blood, the preeminent human biofluid. Sampling and analysis of whole peripheral blood or blood plasma have historically been the gold standard for assessment of physiologic markers of health and disease in clinical diagnostics because, when available, blood samples provide a wealth of systemic analytes.^{14,15} Nonetheless, blood collection is invasive, requires professional training in phlebotomy, can cause anxiety and/or physical discomfort, and is not easily amenable to repeated sampling, particularly in vulnerable populations. When accounting for the constraints of sample collection, processing, and storage in nonclinical populations, saliva sampling is likely preferable to blood for a range of study paradigms.^{2,16} In addition to collection issues, the similar bioavailability of key, physiologically relevant analytes in blood and oral secretions, complemented by improvement in saliva-based assays, suggests that for many different downstream analytes saliva is equivalent to blood or, in many cases, preferable because of the noninvasive nature of collection.^{17,18}

Importance of Methodologic Considerations for Salivary Analytics

There are many potential sources of error when developing a program for salivary diagnostics, in part because salivary analytics is a multistep process of collection, storage, processing, assay, and data analysis. Each step in this process is independent and requires careful consideration of target analyte-dependent issues to prevent undue measurement error, which can multiply in impact within each step.^{19,20} Reliable quality control is paramount to success, and thus issues regarding saliva collection and storage are discussed briefly here.

Sample Collection: Making Choices to Address the Challenges

There is no single gold-standard collection strategy for saliva sampling in human participants, but rather there are a series of choices and challenges inherent to each collection protocol. The particular requirements of individual studies and target subject populations should dictate which sampling method is most appropriate.²¹ Among the choices, for example, saliva can be collected either as stimulated (active secretion) or unstimulated (resting flow during passive drool) saliva. Stimulated whole saliva, derived primarily from the parotid gland, is more dilute and has a distinct protein profile compared with unstimulated saliva. Unstimulated saliva is derived mostly from the submandibular and sublingual glands.^{22,23} Unstimulated saliva, when it can be collected, is generally preferable when target analyte concentrations are low or secreted preferentially under resting conditions. Furthermore, analytes collected in unstimulated saliva that are influenced by flow rate, such as charged corticosteroids (dehydroepiandrosterone sulfate), correlate better with blood concentrations than if the same analyte is assayed in stimulated saliva.²⁴ Although preferable, developmental (eg, young children) and/or overall health considerations (eg, health conditions associated with decreased saliva production) often dictate whether unstimulated saliva collection is practical. Stimulated saliva collection may be desirable when large sample volumes are required and/or target analytes occur in high concentrations. Therefore, preference for sampling of unstimulated or stimulated saliva should account for anticipated downstream applications of the sample and work in favor of the target analytes.^{17,18}

Saliva collection methods also require careful consideration. The decision to use suction versus types of absorbent materials depends on the analyte(s) of interest.^{25,26} Unfortunately, some collection devices may introduce bias and error to downstream assays for particular analytes. For example, assays for immunoglobulins (specific IgA) and certain sex hormones, such as estradiol, can be biased because of the binding of the analytes to absorbent material, thereby requiring the use of suction or passive drool to ensure appropriate fidelity.²⁵ Similarly, use of stabilizing reagents during collection or immediately afterward, as is frequently done with saliva collected for nucleic acids (eg, DNA and RNA), has the benefit of eliminating unwieldy freeze-thaw requirements and stabilizing nucleic acids. However, it also dilutes sample concentrations and alters the chemical composition (eg, electrolytes and pH) of the sample, thus affecting volumetric assays, lowering the analyte concentrations in relation to thresholds of assay sensitivity, and influencing analyte-reporter binding affinity in immunosorbant assays.^{17,18}

To limit sample quality confounds related to common exogenous contaminants of saliva (eg, food or drink residue, medications, and tobacco products), studies should have strict precollection protocols that include water rinses to void all saliva produced outside the clinical setting and instructions to avoid eating or drinking, smoking, or taking medication at least an hour before sampling.^{17,26} Unfortunately, standard practices such as this are not applicable in certain contexts, such as with young children who cannot perform mouth rinses, have irregular feeding schedules, and/or are breastfeeding. When sampling in infants, records should be kept of breastfeeding status, feeding, and sleeping schedules.²⁷ In addition, lack of adult subject adherence to these protocols should be accounted for,

particularly for self-administered collections performed at home or away from the research site.

Rigorous attention to multiple factors related to the saliva collection method is required because of the complex nature of the collection process and must be coordinated, validated, and standardized. In the end, several key factors must be addressed before beginning a study concerned with the correlation of analytes between blood and saliva because each factor can contribute to error and variability: (1) the selection of stimulated versus unstimulated saliva, (2) the long-term requirements for the analyte and downstream analytics, and (3) the type of collection device.²⁰ Below, we consider each of these specific issues in relation to a subset of emerging salivary biomarkers.

INITIAL TARGETS FOR SALIVARY DIAGNOSTICS

Salivary Microbiome

The significant microbial population that colonizes oral cavities is referred to collectively as the salivary microbiome. The existence of a microbiome in saliva distinguishes it from blood and presents both obstacles and opportunities. The preponderance of contaminating prokaryotic biomolecules in saliva, including living organisms and bacterial DNA, RNA, and protein, makes isolating specific human biomolecules challenging, reduces assay sensitivity, and complicates the validation of their origin.³ Efforts to create large-scale data sets describing human biomolecules need to consider analytic approaches that account for the diversity and variability of the salivary microbiome.

Sampling of the salivary microbiome can also provide unique data given that the oral cavity is the first part of the gastrointestinal system and thus provides a unique opportunity to examine the symbiotic relationship between individuals and colonizing microbiota. The balance between bacterial families in the salivary microbiome may also be indicative of more global outcomes beyond oral health.²⁸ Next generation sequencing of microbial (or viral) genetic material present in oral fluids may serve as a unique, independent biomarker of infectious disease.⁸ Bacterial DNA may also be used to identify oral bacterial species or taxa resistant to culturing and other isolation methods.⁹ For instance, pathogenic bacterial load can be quantified from unhealthy dental plaques and caries.²⁹ This emerging research is revealing the unique role of human saliva in systemic immune defense and new insight into the integrity of the immune system within individuals.^{29–31}

In addition to pathologic microbiotic factors, the healthy “core” salivary microbiome can be characterized via deep-sequencing from whole saliva and location-specific dental swabs within the oral cavity, providing additional insight into human health and development.³² For example, salivary microbiome diversity was assessed through sequencing-based approaches within an epidemiologic British population of adults (40 to 69 years old).² In a separate US study, a Human Oral Microbiome Database is being compiled from samples collected from health-screened adults (aged 18 to 40 years) as an independent project in conjunction with the National Institutes of Health–funded Human Microbiome Project.^{28,30} These efforts to characterize the healthy microbiome across different developmental times points will provide essential reference data for studies expecting to explore the role bacteria

play in systemic health. Collectively, this research points out that the salivary microbiome warrants further attention because it represents both a challenge to salivary analytics and a metric of the delicate equilibrium that exists between microbiota-dependent health and pathology.

The Salivary Proteome

The salivary proteome comprises the entire protein content of the oral cavity. Although there is overlap with the blood proteome, the salivary proteome has distinct components subject to specific physiologic regulation within an individual.^{4,33} Currently, the blood proteome is considered more diverse than the salivary proteome, yet >2000 distinct peptides have been found in each.¹⁴ Approximately one-fourth of proteins found in blood plasma have been detected in the oral fluids.⁶ However, although there is substantial overlap in the types of peptides identified in the 2 biological fluids, the relative abundance and distribution differ significantly. Only 22 of >2000 distinct peptides constitute 99% of total blood protein analytes.¹⁴ The overrepresentation of these 22 proteins in blood limits the detectability of lower abundance proteins, such as potential proteomic markers of tumor growth and metastasis. In contrast, the 20 most abundant saliva protein analytes represent less than half (approximately 40%) of the total salivary proteome. Because of the more even distribution of distinct salivary peptides, proteomic analysis in saliva may have distinct advantages over blood, particularly for lower abundance proteins.^{14,33}

Salivary Endocrine Analytes

Although not specifically a component of an “-ome,” this discussion would not be complete without some mention of how endocrine research has contributed significantly to popularizing saliva collection as an alternative to blood sampling. Endocrine measures are particularly relevant to the epigenome because hormones function to alter gene expression, and lipid-soluble corticosteroid hormones do so by traveling through cell membranes directly to the nucleus, where they bind to target DNA sequences triggering gene transcription.¹³ More complex interactions may also exist in the endocrine system because a recent study found an association, in saliva, between acute testosterone reactivity and telomere length in a cohort of African American children (4–14 years old).³⁴

In addition, endocrine measures are relevant because proponents of endocrine salivary diagnostics were some of the first to confront the distinction of saliva from blood and the utility of saliva.^{15,35} A rich literature increasing since the 1990s reports salivary corticosteroid analytes as indirect measures of corticosteroid levels found in blood. Relative concentrations of the principal salivary neuroendocrine bioindicators (cortisol, testosterone, and aldosterone, to name a few) significantly correlate with blood in healthy adults when stringent methodologic controls are used.^{15,19,35} The absolute correspondence between assayable endocrine analyte concentrations in blood and saliva was limited, an initial impediment given that saliva captures only the unbound fraction of total corticosteroid levels, typically 5% to 10% of levels found in blood. As the dynamic nature of the genome has become more appreciated, however, the bioavailability of unbound hormones in saliva has been recast as a potential strength. It is only this biologically active fraction of corticosteroid hormones that is capable of passing through the acinar cells into saliva and

through the cellular and nuclear membranes to bind intracellular receptors and affect gene expression. Thus, saliva may be more informative for corticosteroid assays than blood because quantifiable blood corticosteroid concentration is influenced more readily by binding globulins and carrier proteins.³⁶

Lastly, corticosteroid hormone biomarkers illustrate the benefits of noninvasive collection, particularly repeated sample collection from the same individual over time. Repeated sampling of saliva is usually more practical than blood, thus permitting characterization of diurnal and acute stress reactivity patterns of endocrine function, particularly in vulnerable populations.²⁷ Saliva collection is also less invasive and subsequently less likely to invoke confounding ancillary stress reactivity associated with venipuncture.^{37,38} Protocols with frequent sampling or longitudinal collection strategies illustrated the wide variability inherent in endocrine marker analytics and the substantial fluctuations that these indexes can undergo across a range of time scales. Rather than viewing salivary biomarkers as invariant or static diagnostic entities, fluctuating levels illustrate how the individual can change quickly, especially at a cellular or molecular level. This appreciation of the time-sensitive nature of salivary diagnostics can be perpetuated into more epigenetically informative applications.

SALIVARY “-OMICS” FOR ANALYTICAL DIAGNOSTICS

This next section turns toward an evaluation of the emerging studies exploring the associations of the salivary genome, epigenome, and transcriptome. The following section examines epigenetically informative analytes as we move beyond a static view of genomic cellular regulation toward a conceptualization of epigenomic factors as development-dependent markers that may fluctuate to affect health across the lifespan.

The Salivary DNA

It has been established that high-quality, high-molecular-weight DNA molecules can be purified from human saliva, which will be collectively referred to as the salivary genome. Using an expanding range of DNA collection kits, stabilizing reagents, and purification procedures developed for saliva samples,²⁰ the yield and quality of both saliva and blood-derived DNA are comparable.¹⁶ Both blood and saliva yield DNA that can be reliably applied to a diverse array of down-stream applications, but given the ease of sampling and reliability of assays, saliva-based DNA analysis has benefited from appreciation and application on population-wide scales.² Perhaps given this unprecedented popularity, as well as the widespread appreciation of the time-varying nature of markers for health (eg, immunoglobulins) and development (eg, hormones), the field is now moving beyond a static view of the salivary genome to include time-sensitive and context-dependent epigenomic elements. These elements are assayable by a diverse collection of powerful biomolecular techniques, including methylation arrays and a range of PCR and qPCR-based approaches for genotyping and telomere length assays.^{10,39–41} Some of these innovative methods are addressed below.

The Salivary Epigenome

Whereas the genome is the template for RNA transcription and ultimately controls functional expression of relevant proteins, the epigenome highlights how the genome itself is environmentally regulated and capable of adaptation over time through chemical and structural modification that does not alter the DNA basepair sequence. The epigenome is often probed via DNA methylation, although there are further environment-dependent epigenetic changes measurable with saliva (eg, telomere length and micro-RNAs [miRNAs]). Epigenetic changes between salivary and blood nucleic acids are thought to reflect differences in the tissue and cell type of origin. Blood DNA is derived from a diverse population of immune cells that can vary significantly over time, dependent on individual states of immune activation. The cellular components of saliva, however, are representative of polymorphonucleated cells, including basophils, neutrophils, and eosinophils, as well as shed epithelial cells from the lining of the oral cavity. Furthermore, previous studies indicate that most saliva DNA originates from this much more homogenous subpopulation of polymorphonucleated leukocytes, contrary to DNA purified from whole blood.^{42,43} Thus, blood and saliva are not expected to have high correspondence, and there are valid arguments for why saliva is, for many health outcomes, more advantageous than a blood-based approach within epigenetic research.

Salivary DNA Methylation

Methylation and demethylation of genes and promoters of genes, respectively, are 2 central epigenetic mechanisms that regulate gene expression patterns across tissues and developmental set points.⁴⁴ Methylation involves the addition of a methyl group to DNA nucleotides, thus influencing structural organization and the binding of transcription factors. Salivary DNA has been used to study methylation changes in relation to systemic conditions, such as diabetes⁴⁵ or a type of muscular dystrophy,⁴⁶ and early life adversity and maltreatment.⁴⁷ As a marker of developmental variance, analysis of methylation profiles in saliva revealed methylation states of many target genes to be sex specific. Importantly, subsequent validation of these findings using blood samples indicated that DNA sourced from saliva and blood are comparable.³⁹ In a comparative genome-wide methylation study of whole blood DNA, saliva DNA, and DNA-derived from cultured lymphoblastoid cell lines, blood and saliva genomic methylation patterns were again found to be comparable in 14 healthy adults.⁴¹ Salivary DNA methylation patterns correlate with both blood and central nervous tissue across different regions of the brain in a group of African American adults. In fact, methylation patterns in the brain, across different regions, were more correlated with the saliva DNA methylation than blood methylation.⁴⁸ These results are further supported by studies revealing clear correspondence between methylation profiles in the brain and saliva of young, healthy adult subjects,⁴⁹ as well as in clinical populations (eg, adults with schizophrenia).^{50–52}

DNA methylation is an important part of the salivary epigenome because it reflects the mechanisms through which gene expression changes over time along with how environmental cues may affect behavioral adaptations. Exposure to adversity early in life is one possible factor that mediates epigenetic modification of the genome. Experiences of chronic violence or neglect, characteristic of early adversity, have been reported to influence

the methylation state of important neuropsychiatric gene targets. In one such study of abused children aged 5 to 14 years, saliva samples were collected to measure changes in DNA methylation patterns. A genome-wide methylation assay of maltreated children versus controls using saliva-derived DNA revealed a significant alteration of methylation pattern throughout the genome related to chronic experiences of abuse and associated stressors.⁵³

Salivary Telomere Length

Whereas DNA methylation is readily described as a functional component of the epigenome, it is now recognized that telomeres, the distal segments of nucleotide repeats that form stabilizing caps at the ends of chromosomes, are also a dynamic element of the functional genome that change over time. Recent methodologic innovations have made it possible to measure the telomere length of human DNA isolated from the salivary genome using monochrome multiplex qPCR.⁵⁴ Such an assay has the potential to establish salivary telomere length (sTL) as a direct marker of genome stability and maintenance within individuals or populations of human subjects exposed to any number of physical, chemical, or psychosocial stressors. sTL has been successfully measured in both adult⁵⁵ and child^{10–12} cohorts in a variety of environment-dependent contexts, ranging from social stress,⁵⁵ community disorder,¹¹ and social disadvantage¹² to tobacco exposure.^{10,55} For example, salivary genomic DNA was purified and used to determine that sTL in children of women who smoked tobacco during pregnancy were substantially shorter than children unexposed to prenatal tobacco.¹⁰ Similarly, children from that study cohort were also found to have significantly reduced sTL when living in neighborhoods characterized by high disorder.¹¹ In a related study by Mitchell et al,¹² social disadvantage in childhood was found to predict shortened sTL. Notably, this group also reported a significant correlation between sTL and blood leukocyte DNA telomere length in a small group of healthy adult subjects.¹² Further validation of sTL may establish it as a robust marker of cellular aging and predictor of negative health outcomes.

The Salivary Transcriptome

The salivary transcriptome is the cumulative pool of RNA species found in saliva. A diverse salivary transcriptome has been isolated consistently from saliva samples using a range of validated methods and has been extensively characterized.⁵⁶ Furthermore, complete characterization of the salivary transcriptome has revealed a high degree of overlap with blood RNA, as well as a unique profile of saliva-specific mRNA and microRNA transcripts. Although the techniques to examine the salivary transcriptome are now well developed, the translational development of salivary transcriptomics for clinical applications remains in its infancy.^{3,57,58}

Salivary mRNA isolation kits have now become readily available from a number of commercial entities, such as Qiagen (RNeasy Protect Saliva) and DNA Genotek (Oragene Saliva RNA). Adapting one of these commercially available kits, Li et al⁵⁹ were the first to successfully isolate human mRNA from cell-free supernatant saliva. The profile of purified salivary RNA was evaluated by high-density microarrays and confirmed by qRT-PCR assay, identifying approximately 3000 mRNA transcripts.⁵⁹ Furthermore, the salivary transcriptome was unexpectedly stable despite a high prevalence of moderate degradation

and evidence that saliva is rich in endogenous ribonucleases.^{56,59,60} In vitro experiments suggest that extracellular mRNA transcripts interact with endogenous stabilization factors to attenuate the rate of salivary mRNA degradation.⁵ Notwithstanding initial skepticism concerning the validity of a cell-free salivary transcriptome,⁶¹ extensive characterization of purified mRNA from pooled supernatant human saliva, using parallel sequencing, has provided a definitive confirmation of efficacy for saliva-based mRNA extraction and expression studies.^{3,58,62}

Identification of Salivary Transcriptomic Biomarkers of Disease

Saliva-based cancer diagnostics have proven to be fertile ground for research and development relative to changing mRNA profiles of the salivary transcriptome. Examples of the translational utility of salivary transcriptomics have been described previously in vitro,⁶³ within animal models,⁵⁷ and for human patients,⁵⁸ revealing a potential role for salivary gland secretion profiling in the diagnosis of nonlocalized cancers. One such in vitro study found that breast cancer cells release microvesicles that interact with salivary gland cells to modify intracellular transcription, exocytotic pathways, and salivary microvesicle composition.⁶³ Similarly, exosomes released by pancreatic tumors in a rodent model triggered identifiable, disease-specific differences in the salivary transcriptome. The importance of this work was 2-fold. First, this study revealed the utility of the salivary transcriptome in identifying the presence of pancreatic tumors. Second, this study found that the observed changes in the salivary transcriptome were the result of exosomes released from the tumors themselves. The identified transcriptional changes were specific to saliva and not replicated in the blood.⁵⁷ In rodents, both melanoma and non-small cell lung cancer caused systemic release of growth factors that altered expression and secretion for a subset of transcription factors from salivary glands.⁶⁴

In humans, high sensitivity and specificity for head and neck cancer identification have been achieved through analyses of specific salivary transcriptomic markers. Direct comparison of oral cancer biomarkers between the salivary and blood transcriptomes suggests substantial utility for salivary analytics independent of blood-based approaches. The validity of cancer-related components of the transcriptome as diagnostic and prognostic markers of oncogenesis further supports the unique potential of saliva. As more salivary biomarkers of cancer and other illnesses are identified and validated in vitro or with animal models, the clinical efficacy of salivary diagnostics is expected to increase.⁴⁴

MicroRNA Expression

Additional epigenetic factors, such as noncoding RNAs (ncRNAs), are increasingly recognized for their experience-dependent responsivity and have been characterized in saliva by microarray and sequencing-based techniques. ncRNA species are a diverse family of RNA molecules that do not serve as gene templates for protein translation. Several classes of ncRNAs exist, including long ncRNA, small nucleolar RNA, and miRNA, all of which have been detected in saliva. The salivary miRNAome may be of particular relevance for biomarker-based investigation because of its role in epigenetic modulation of cellular adaptation over time.³

Mature miRNAs are short (18 to 25 nucleotides) noncoding transcripts that bind complementary sequences within target mRNA transcripts and modify translation turnover through association with complementary proteins collectively known as RNA-induced silencing complexes. RNA-induced silencing complexes function to inhibit or delay mRNA-ribosome binding and promote sequestration or degradation of captured mRNA. Therefore, miRNAs function to dynamically control cellular activity through posttranscriptional regulation of miRNA-dependent silencing.⁶⁵ Saliva appears to contain a unique spectrum of miRNA species,^{66,67} and global analysis of the salivary miRNAome has revealed a well-characterized, comprehensive profile of saliva-specific expression.⁶⁸

Salivary miRNAs are both abundant and relatively stable. Native salivary miRNA is significantly more stable in saliva than exogenously applied miRNA, suggesting that endogenous miRNAs complex with protective factors, similar to salivary mRNA. A protein component of the RNA-induced silencing complex, Ago 2, is found in supernatant saliva and may contribute to salivary miRNA stability.⁶⁵ Packaging of salivary miRNA within exosomal structures likely provides further protection from extracellular ribonucleases. The exosomal fraction purified from whole saliva is enriched with miRNAs compared with whole unfractionated saliva and the exosome-depleted supernatant saliva.⁶⁹ Exosomally derived miRNAs have been isolated and extensively profiled in an effort to identify disease biomarkers in adult patients with Sjögren syndrome, an autoimmune disease that affects the salivary glands.⁷⁰

Salivary miRNA expression studies have both research and clinical applications for forensics,⁷¹ oral cancer,⁶⁵ autoimmune disease,⁷⁰ and physioanatomical development in children.⁷² For instance, a small subset of miRNAs are potential diagnostic markers of cancer in the saliva of patients with oral squamous cell carcinoma.⁶⁵ There is also evidence that salivary miRNAome expression changes in relation to normal development. Developmental differences in the expression of certain miRNAs was found in healthy children aged 2 months to 13 years and appear to be epigenetic indications reflective of dentition stages and oral maturation.⁷² Appreciation of developmental and environmental influence on miRNA expression profiles is an important next step in human salivary analytics.

CONCLUSION

This article describes the utility and practicality of salivary diagnostics. We first sought to delineate the current state of salivary analytics and biomarker development with a critical view of the approaches and applications of saliva-based methods. This critical lens notwithstanding, it is clear that there is utility in salivary analytics, especially when methodologic issues are addressed in the initial study design. Sample quality, assay sensitivity, and analytical validity are equal, or in some cases superior, to that of blood-based diagnostics for an increasing number of biomolecular targets and analytes. The ability of this noninvasive biofluid to provide time-sensitive information uniquely positions saliva researchers to extend the range of diagnostic applications with ongoing and emerging innovations already apparent relative to the salivary microbiome, proteome, transcriptome, and epigenome. Changes within these “salivaomics”⁷³ are not random but appear to be

experience dependent and potentially informative about an individual's prior developmental history, as well as both current and subsequent health-related risks. The challenge for the field now is to confront how best to translate these readily available analytic approaches in salivary diagnostics to predict an individual's long-term health and risk.

Acknowledgments

The first author conducted the initial literature review and composed the preliminary draft. All authors contributed equally to all subsequent writing, revision and editing of the manuscript. None received any financial compensation for their preparation of this manuscript, and they are solely responsible for the content.

References

1. Fabian TK, Fejerdy P, Csermely P. Salivary genomics, transcriptomics and proteomics: The emerging concept of the oral ecosystem and their use in the early diagnosis of cancer and other diseases. *Curr Genomics*. 2008; 9:11–21. [PubMed: 19424479]
2. Pramanik R, et al. Effects of the UK Biobank collection protocol on potential biomarkers in saliva. *Int J Epidemiol*. 2012; 41:1786–1797. [PubMed: 23148109]
3. Spielmann N, et al. The human salivary RNA transcriptome revealed by massively parallel sequencing. *Clin Chem*. 2012; 58:1314–1321. [PubMed: 22773539]
4. Amado FM, Ferreira RP, Vitorino R. One decade of salivary proteomics: current approaches and outstanding challenges. *Clin Biochem*. 2013; 46:506–517. [PubMed: 23103441]
5. Park NJ, et al. Characterization of RNA in saliva. *Clin Chem*. 2006; 52:988–994. [PubMed: 16601067]
6. Spielmann N, Wong DT. Saliva: diagnostics and therapeutic perspectives. *Oral Dis*. 2011; 17:345–354. [PubMed: 21122035]
7. Humphrey SP, Williamson RT. A review of saliva: normal composition, flow, and function. *J Prosthet Dent*. 2001; 85:162–169. [PubMed: 11208206]
8. Yoshizawa JM, et al. Salivary biomarkers: toward future clinical and diagnostic utilities. *Clin Microbiol Rev*. 2013; 26:781–791. [PubMed: 24092855]
9. Malamud D, Rodriguez-Chavez IR. Saliva as a diagnostic fluid. *Dent Clin North Am*. 2011; 55:159–178. [PubMed: 21094724]
10. Theall KP, et al. Early hits and long-term consequences: tracking the lasting impact of prenatal smoke exposure on telomere length in children. *Am J Public Health*. 2013; 103 (Suppl 1):S133–S135. [PubMed: 23927510]
11. Theall KP, et al. Neighborhood disorder and telomeres: connecting children's exposure to community level stress and cellular response. *Soc Sci Med*. 2013; 85:50–58. [PubMed: 23540366]
12. Mitchell C, et al. Social disadvantage, genetic sensitivity, and children's telomere length. *Proc Natl Acad Sci U S A*. 2014; 111:5944–5949. [PubMed: 24711381]
13. Meaney MJ, Szyf M, Seckl JR. Epigenetic mechanisms of perinatal programming of hypothalamic-pituitary-adrenal function and health. *Trends Mol Med*. 2007; 13:269–277. [PubMed: 17544850]
14. Loo JA, et al. Comparative human salivary and plasma proteomes. *J Dent Res*. 2010; 89:1016–1023. [PubMed: 20739693]
15. Vining RF, McGinley RA, Symons RG. Hormones in saliva: mode of entry and consequent implications for clinical interpretation. *Clin Chem*. 1983; 29:1752–1756. [PubMed: 6225566]
16. Hansen TV, et al. Collection of blood, saliva, and buccal cell samples in a pilot study on the Danish nurse cohort: comparison of the response rate and quality of genomic DNA. *Cancer Epidemiol Biomarkers Prev*. 2007; 16:2072–2076. [PubMed: 17932355]
17. Granger DA, et al. Incorporating Salivary Biomarkers Into Nursing Research: An Overview and Review of Best Practices. *Biol Res Nurs*. 2012; 14:347–356. [PubMed: 22593229]

18. Granger DA, et al. Integration of salivary biomarkers into developmental and behaviorally-oriented research: Problems and solutions for collecting specimens. *Physiol Behav.* 2007; 92:583–590. [PubMed: 17572453]
19. Granger DA, et al. Focus on Methodology: Salivary bioscience and research on adolescence: An integrated perspective. *J Adolesc.* 2012; 35:1081–1095. [PubMed: 22401843]
20. Slowey, PD. Saliva Collection Devices and Diagnostic Platforms, in *Advances in Salivary Diagnostics*. Streckfus, C., editor. Berlin Heidelberg: Springer-Verlag; 2015.
21. Slowey PD. Commercial saliva collections tools. *J Calif Dent Assoc.* 2013; 41:97–99. [PubMed: 23505755]
22. del de Almeida PV, et al. Saliva composition and functions: a comprehensive review. *J Contemp Dent Pract.* 2008; 9:72–80. [PubMed: 18335122]
23. Navazesh M, Kumar SKS. Measuring salivary flow -Challenges and opportunities. *J Am Dent Assoc.* 2008; 139:35S–40S. [PubMed: 18460678]
24. Lewis JG. Steroid analysis in saliva: an overview. *Clin Biochem Rev.* 2006; 27:139–146. [PubMed: 17268582]
25. Shirtcliff EA, et al. Use of salivary biomarkers in bio-behavioral research: cotton-based sample collection methods can interfere with salivary immunoassay results. *Psychoneuroendocrinology.* 2001; 26:165–173. [PubMed: 11087962]
26. Navazesh M. Methods for collecting saliva. *Ann N Y Acad Sci.* 1993; 694:72–77. [PubMed: 8215087]
27. Tryphonopoulos PD, Letourneau N, Azar R. Approaches to salivary cortisol collection and analysis in infants. *Biol Res Nurs.* 2014; 16:398–408. [PubMed: 24136995]
28. Human Microbiome Project, C. A framework for human microbiome research. *Nature.* 2012; 486:215–221. [PubMed: 22699610]
29. Nadkarni MA, et al. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology-Sgm.* 2002; 148:257–266.
30. Chen T, et al. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database (Oxford).* 2010;baq013. [PubMed: 20624719]
31. Yan WH, et al. Systematic comparison of the human saliva and plasma proteomes. *Proteomics Clin Appl.* 2009; 3:116–134. [PubMed: 19898684]
32. Zaura E, et al. Defining the healthy “core microbiome” of oral microbial communities. *BMC Microbiol.* 2009; 9:259. [PubMed: 20003481]
33. Pfaffe T, et al. Diagnostic potential of saliva: current state and future applications. *Clin Chem.* 2011; 57:675–687. [PubMed: 21383043]
34. Drury SS, et al. Growing up or growing old? Cellular aging linked with testosterone reactivity to stress in youth. *Am J Med Sci.* 2014; 348:92–100. [PubMed: 25010187]
35. Chiappin S, et al. Saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clin Chim Acta.* 2007; 383:30–40. [PubMed: 17512510]
36. Gozansky WS, et al. Salivary cortisol determined by enzyme immunoassay is preferable to serum total cortisol for assessment of dynamic hypothalamic–pituitary–adrenal axis activity. *Clin Endocrinol (Oxf).* 2005; 63:336–341. [PubMed: 16117823]
37. Marceau K, Dorn LD, Susman EJ. Stress and puberty-related hormone reactivity, negative emotionality, and parent–adolescent relationships. *Psychoneuroendocrinology.* 2012; 37:1286–1298. [PubMed: 22284540]
38. Marceau K, et al. Within-adolescent coupled changes in cortisol with DHEA and testosterone in response to three stressors during adolescence. *Psychoneuroendocrinology.* 2014; 41:33–45. [PubMed: 24495606]
39. Liu JY, et al. A Study of the Influence of Sex on Genome Wide Methylation. *Plos One.* 2010;5.
40. Nemoda Z, et al. Assessing genetic polymorphisms using DNA extracted from cells present in saliva samples. *BMC Med Res Methodol.* 2011; 11:170. [PubMed: 22182470]
41. Thompson TM, et al. Comparison of whole-genome DNA methylation patterns in whole blood, saliva, and lymphoblastoid cell lines. *Behav Genet.* 2013; 43:168–176. [PubMed: 23269419]

42. Endler G, et al. Genetic fingerprinting in mouthwashes of patients after allogeneic bone marrow transplantation. *Bone Marrow Transplant.* 1999; 24:95–98. [PubMed: 10435742]
43. Thiede C, et al. Buccal swabs but not mouthwash samples can be used to obtain pretransplant DNA fingerprints from recipients of allogeneic bone marrow transplants. *Bone Marrow Transplant.* 2000; 25:575–577. [PubMed: 10713640]
44. Bonne NJ, Wong DT. Salivary bio-marker development using genomic, proteomic and metabolomic approaches. *Genome Med.* 2012; 4:82. [PubMed: 23114182]
45. Sapienza C, et al. DNA methylation profiling identifies epigenetic differences between diabetes patients with ESRD and diabetes patients without nephropathy. *Epigenetics.* 2011; 6:20–28. [PubMed: 21150313]
46. Jones TI, et al. Identifying diagnostic DNA methylation profiles for facioscapulohumeral muscular dystrophy in blood and saliva using bisulfite sequencing. *Clin Epigenetics.* 2014; 6:23. [PubMed: 25400706]
47. Weder N, et al. Child abuse, depression, and methylation in genes involved with stress, neural plasticity, and brain circuitry. *J Am Acad Child Adolesc Psychiatry.* 2014; 53:417–424. [PubMed: 24655651]
48. Smith AK, et al. DNA extracted from saliva for methylation studies of psychiatric traits: Evidence tissue specificity and relatedness to brain. *Am J Med Genet B Neuropsychiatr Genet.* 2014; 9999B:1–9.
49. Nikolova YS, et al. Beyond genotype: serotonin transporter epigenetic modification predicts human brain function. *Nat Neurosci.* 2014; 17:1153–1155. [PubMed: 25086606]
50. Abdolmaleky HM, et al. DNA hypermethylation of serotonin transporter gene promoter in drug naive patients with schizophrenia. *Schizophr Res.* 2014; 152:373–380. [PubMed: 24411530]
51. Ghadirivasfi M, et al. Hypomethylation of the serotonin receptor type-2A Gene (HTR2A) at T102C polymorphic site in DNA derived from the saliva of patients with schizophrenia and bipolar disorder. *Am J Med Genet B Neuropsychiatr Genet.* 2011; 156B:536–545. [PubMed: 21598376]
52. Nohesara S, et al. DNA hypomethylation of MB-COMT promoter in the DNA derived from saliva in schizophrenia and bipolar disorder. *J Psychiatr Res.* 2011; 45:1432–1438. [PubMed: 21820670]
53. Yang BZ, et al. Child abuse and epigenetic mechanisms of disease risk. *Am J Prev Med.* 2013; 44:101–107. [PubMed: 23332324]
54. Cawthon RM. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. *Nucleic Acids Res.* 2009; 37:e21. [PubMed: 19129229]
55. Chen X, et al. Smoking and perceived stress in relation to short salivary telomere length among caregivers of children with disabilities. *Stress.* 2014;1–9.
56. Park NJ, et al. Characterization of salivary RNA by cDNA library analysis. *Arch Oral Biol.* 2007; 52:30–35. [PubMed: 17052683]
57. Lau C, et al. Role of pancreatic cancer-derived exosomes in salivary biomarker development. *J Biol Chem.* 2013; 288:26888–26897. [PubMed: 23880764]
58. Segal A, Wong DT. Salivary diagnostics: enhancing disease detection and making medicine better. *Eur J Dent Educ.* 2008; 12(Suppl 1):22–29. [PubMed: 18289265]
59. Li Y, et al. RNA Profiling of Cell-free Saliva Using Microarray Technology. *J Dent Res.* 2004; 83:199–203. [PubMed: 14981119]
60. Eichel HJ, Conger N, Chernick WS. Acid and Alkaline Ribonucleases of Human Parotid, Submaxillary, and Whole Saliva. *Arch Biochem Biophys.* 1964; 107:197–208. [PubMed: 14224825]
61. Kumar SV, Hurteau GJ, Spivack SD. Validity of messenger RNA expression analyses of human saliva. *Clin Cancer Res.* 2006; 12:5033–5039. [PubMed: 16951218]
62. Zimmermann BG, Park NJ, Wong DT. Genomic targets in saliva. *Ann N Y Acad Sci.* 2007; 1098:184–191. [PubMed: 17435127]
63. Lau CS, Wong DT. Breast cancer exosome-like microvesicles and salivary gland cells interplay alters salivary gland cell-derived exosome-like microvesicles in vitro. *PLoS One.* 2012; 7:e33037. [PubMed: 22448232]

64. Gao K, et al. Systemic Disease-Induced Salivary Biomarker Profiles in Mouse Models of Melanoma and Non-Small Cell Lung Cancer. *Plos One*. 2009;4.
65. Park NJ, et al. Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection. *Clin Cancer Res*. 2009; 15:5473–5477. [PubMed: 19706812]
66. Hanson EK, Lubenow H, Ballantyne J. Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs. *Anal Biochem*. 2009; 387:303–314. [PubMed: 19454234]
67. Zubakov D, et al. MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation. *Int J Legal Med*. 2010; 124:217–226. [PubMed: 20145944]
68. Weber JA, et al. The microRNA spectrum in 12 body fluids. *Clin Chem*. 2010; 56:1733–1741. [PubMed: 20847327]
69. Gallo A, et al. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. *PLoS One*. 2012; 7:e30679. [PubMed: 22427800]
70. Michael A, et al. Exosomes from human saliva as a source of micro-RNA biomarkers. *Oral Dis*. 2010; 16:34–38. [PubMed: 19627513]
71. Courts C, Madea B. Specific micro-RNA signatures for the detection of saliva and blood in forensic body-fluid identification. *J Forensic Sci*. 2011; 56:1464–1470. [PubMed: 21827476]
72. Formosa A, et al. Salivary miRNAome profiling uncovers epithelial and proliferative miRNAs with differential expression across dentition stages. *Cell Cycle*. 2011; 10:3359–3368. [PubMed: 21926479]
73. Ai JY, Smith B, Wong DT. Bioinformatics advances in saliva diagnostics. *Int J Oral Sci*. 2012; 4:85–87. [PubMed: 22699264]