

# The Emerging Landscape of Salivary Diagnostics

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## Abstract

Saliva as a diagnostic tool provides a noninvasive, simple and low-cost method for disease detection and screening. Saliva collection is more practical and comfortable compared with other invasive methods, and saliva can be a desirable body fluid for biomarker detection in clinical applications. The integration of omics methods has allowed accurate detection and quantification of transcripts found in saliva and a group of biomarkers has been discovered and validated in a series of studies. Here we review recent developments in salivary diagnostics that have been accomplished using salivaomics, the mechanisms of saliva diagnostics, as well as the translational and clinical application of saliva biomarkers.

*Key Words: Salivary diagnostics, Salivaomics, Mechanisms, Translational and clinical applications*

**Abbreviations:** mRNA: Messenger RNA; miRNA: Micro RNA; PCR: Polymerase Chain Reaction; KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; MBD3L2: Methyl-CpG binding domain protein 3-like 2; ACRV1: Acrosomal vesicle protein 1; DPM1: Dolichyl-phosphate mannosyltransferase polypeptide 1; qRT-PCR: Quantitative reverse transcriptase PCR; MNDA: Myeloid cell nuclear differentiation antigen; GBP-2: Guanylate binding protein 2, interferoninducible; FCGR3B: Low-affinity IIIb receptor for the Fc fragment of IgG; HP: haptoglobin; AZGP1: zinc $\alpha$ 2-glycoprotein; Egr-1: Early growth response protein 1; HER-2: human epidermal growth factorreceptor-2; IL: interleukin; SAT: Spermidine/spermine N1-acetyltransferase; OAZ1: Ornithine decarboxylase antizyme 1; H3F3A: H3 histone, family 3A; DUSP: Dual specificity phosphatase 1; S100P: S100 calcium binding protein P.

## Introduction

High-morbidity and high-mortality diseases such as cancer and other chronic diseases such as cardiovascular, metabolic, and neurological diseases are difficult to diagnose without supplemental laboratory testing. The earlier a disease is detected and diagnosed, the more likely appropriate treatment will be administered to reduce the severity of the disease on the patient. Early detection is therefore urgent for clinical treatment. However, three limitations prevent the realization of the full benefits of clinical diagnostics: definitive biomarkers associated with disease; simple and inexpensive methods that are minimally invasive; and an accurate, portable, and easy-to-use diagnostic platform.

Saliva is a biofluid secreted by salivary glands (the parotid, submandibular, sublingual, and other minor salivary glands). Similar to serum and other biofluids, saliva contains biomolecules such as DNA, mRNA, miRNA, protein, metabolites, and microbiota (*Figure 1*). Because obtaining

saliva is low-cost, noninvasive, simple, and does not cause discomfort for the patient, it can be a highly desirable body fluid for biomarker development for clinical applications. The aim of this review is to provide a status review of salivary “omics” constituents, salivary diagnostics, and their translational and clinical applications.

## Salivaomics

Saliva contains a variety of biomolecules, including DNA, mRNA, miRNA, protein, metabolites, and microbiota, and we can detect changes in their salivary concentration to develop dysregulated biomarkers to detect early oral and systemic diseases, evaluate disease prognosis and risk, and monitor the response to treatment [1,2]. The term “salivaomics” was coined in 2008 to reflect the knowledge about the various “omics” constituents in saliva, including the genome, epigenome, transcriptome, proteome, metabolome, and microbiome [3,4].

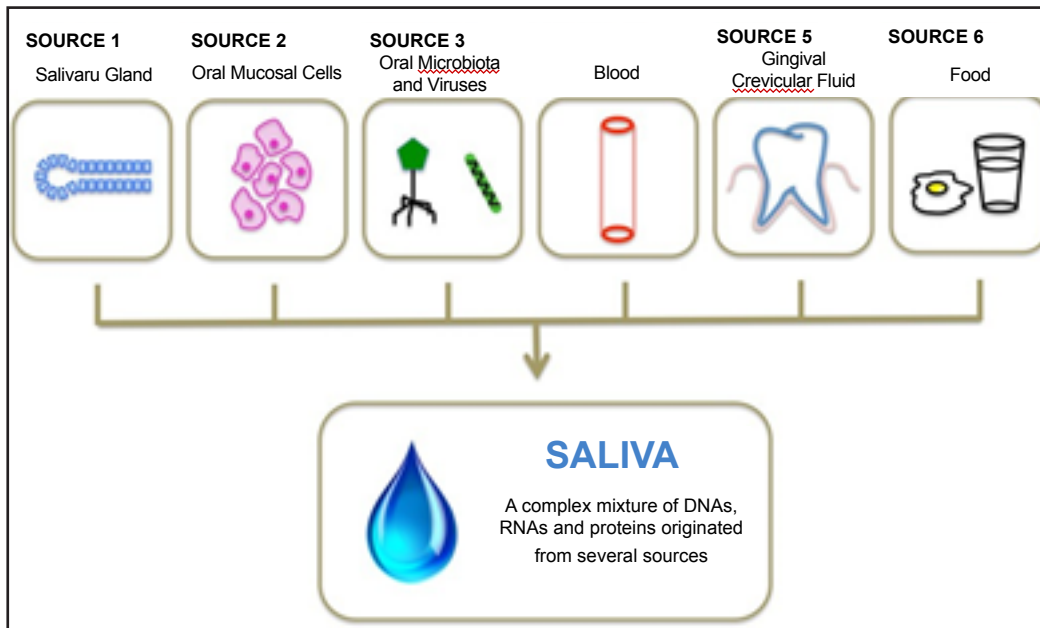
### The salivary genome and epigenome

The salivary genome consists of both human and microbiome DNAs. Nearly 70% of the salivary genome is of human origin, while the remaining 30% is from the oral microbiota [5]. The quality of salivary DNA is good: 72% to 96% of samples can be genotyped; 84% can be amplified; and 67% can be sequenced [6,7] and can be stored long-term without significant degradation [8]. Salivary genetic and epigenetic analysis provides abnormal gene transcription profiles that reflect pathological genetic processes such as Oral Squamous Cell Carcinoma (OSCC) [9,10].

DNA methylation is an epigenetic process that can change in response to the passage of time, development, or environmental exposure [11]. Aberrant methylation of genes (e.g., promoter hypermethylation) is common in cancers [12,13].

Viet et al. [10] completed a methylation array on DNA extracted from preoperative saliva, postoperative saliva, and

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*Figure 1. Biomolecules and fluids from different sources constitute the saliva.*

tissue from patients with OSCC, as well as saliva from healthy subjects. They found significant differences in methylation patterns between the preoperative and postoperative saliva of cancer patients, and between preoperative saliva from OSCC patients and saliva from healthy controls. Gene panels of 4 to 10 genes were constructed and exhibited a sensitivity of 62% to 77% and a specificity of 83% to 100% for OSCC. Carvalho et al. [9] evaluated tumor suppressor gene promoters in pretreatment saliva from patients with Head and Neck Squamous Cell Carcinoma (HNSCC) by using Quantitative Methylation-specific PCR (Q-MSP); more than 50% of patients demonstrated methylation of at least one of the selected genes in the saliva DNA, and local disease control and overall survival were significantly lower in patients presenting with hypermethylation in saliva rinses.

#### **Transcriptomes (mRNA and miRNA)**

Transcriptome studies have focused mainly on mRNA and miRNA, which are secreted from cells and enter the oral cavity through various sources, including salivary glands, gingival crevice fluid, and desquamated oral epithelial cells [14]. The transcription of specific mRNA and miRNA is altered in disease states. Recent research has revealed more than 3,000 species of mRNA in saliva of healthy subjects [15] and 314 of the 708 human miRNAs registered in the miRBase were profiled in the saliva of healthy subjects and OSCC patients [16], suggesting that transcriptomic analysis can be of value to monitor healthy and disease states.

The human salivary transcriptome was first discovered in our laboratory using microarray technology, allowing high-throughput analysis [15]. We then developed Direct-Saliva-Transcriptomic-Analysis (DSTA) to permit simple stabilization of salivary RNA and direct analysis without further processing [17,18].

During the past 5 years, we have reported the detection of salivary mRNA biomarkers in a number of cancers and systemic diseases. Using the Affymetrix HG-U133-Plus-2.0 array for discovery and qRT-PCR for validation, Zhang et al. [19] identified four mRNA biomarkers (KRAS, MBD3L2, ACRV1, and DPM1) that can differentiate early

stage resectable pancreatic cancer patients from non-cancer subjects (chronic pancreatitis patients and healthy controls) with high sensitivity and specificity. Aside from pancreatic cancer, results from our laboratory further demonstrated the utility of salivary mRNAs for the detection of oral cancer [20], Lung Cancer [21], breast cancer [22], ovarian cancer [23], and other systemic diseases. Hu et al. [24] reported that three mRNA biomarkers (MNDA, GBP-2, and FCGR3B) were significantly elevated in patients with primary Sjögren's syndrome compared with both Systemic Lupus Erythematosus (SLE) patients and healthy controls.

miRNA is a group of small noncoding RNAs (19 to 25 nucleotides) that are encoded by genes but are not translated into proteins. miRNAs are centrally involved in various biological processes, including cell differentiation, proliferation, and survival [25]. Many studies have shown that miRNA is frequently dysregulated in cancer tissues compared with healthy controls [26-28].

Compared with salivary mRNA, salivary miRNA are more stable [16,29], and the fold change in miRNA between cancer and normal cells is fairly large [30,31]. Park et al. [16] used reverse transcriptase-preamplification-quantitative PCR to measure salivary miRNAs from OSCC patients and healthy controls. They found that two miRNAs (miR-125a, miR-200a) in the saliva of OSCC patients were significantly reduced compared with healthy controls. Matse et al. [32] investigated differences of miRNA expression in saliva from patients malignant and benign parotid gland tumors using the Taqman miRNA array cards for discovery and qRT-PCR for the validation phase. Their results indicated that a combination of four miRNAs (hsa-miR-132, hsa-miR-15b, mmu-miR-140, and hsa-miR-223) is valuable in the detection of parotid gland malignancy.

To advance saliva extracellular RNA biomarkers discovery, massive parallel sequencing of transcripts (RNA-Seq) was used to sequence and characterize the salivary transcriptome in greater detail [33]. RNA-Seq is analytically more sensitive than microarrays, and can detect differentially expressed genes and provides information about each RNA

sequence that we were unable to obtain in previous studies [34,35]. Approximately 20% to 25% of the sequenced reads from Cell-free Saliva (CFS) correspond with the human genome, and approximately 30% of the sequenced reads correspond with the Human Oral Microbiome Database (HOMD). More than 4000 coding and non-coding genes in CFS and Whole Saliva (WS) were detected [36].

### The proteome

Saliva contains more than 2,000 proteins that are involved in many biological functions to maintain oral homeostasis [37]. Unlike the relatively stable status in serum, proteins in saliva appear to be more susceptible to biochemical processes and degradation [38,39]. Esser et al. [40] reported that salivary protein degradation happens rapidly, and even happens during saliva collection and handling, which may compromise its clinical usefulness. Our laboratory has developed methods to stabilize the salivary proteome with protease inhibitors; as a result, we can keep salivary proteins stable for 2 weeks when stored at 4°C without significant degradation and without affecting downstream applications [41].

Because of high-sensitivity and high-accuracy mass measurement of peptides, Mass Spectrometry (MS) has become the core technologies for protein identification. Surface-enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF MS) has proven to be a powerful tool to identify and quantify Post-Translational Modifications (PTMs) on proteins in saliva. Several studies have reported biomarkers in saliva using this rapid and high-throughput tool [42-44]. Two-dimensional Difference Gel Electrophoresis (2D-DIGE) combined with MS has recently been used in salivary proteomic biomarker discoveries. Using 2D-DIGE, Hu et al. [45,46] reported 16 peptides in saliva that were found at significantly different levels in patients with primary Sjögren's syndrome compared with healthy controls. Xiao et al. [47] reported three proteins (HP, AZGP1, and human calprotectin) that had good discriminatory power in Lung Cancer patients and healthy control subjects, with high sensitivity (89%) and high specificity (92%). Studies also detected salivary protein biomarkers in chronic periodontitis [48], oral cancer [49], and tongue cancer [50].

### The metabolome

The metabolome, which enables the parallel evaluation of a group of endogenous and exogenous metabolites, including lipids, amino acids, peptides, nucleic acids, organic acids, vitamins, thiols, and carbohydrates, is a valuable tool for discovering biomarkers, monitoring physiological status, and making proper treatment decisions [51-53].

Based on the different metabolomic technology, studies have reported salivary metabolites cannot only identify health status [54,55], but can also discriminate diseased patients from healthy control subjects. Sugimoto et al. [56] used Capillary Electrophoresis Time-of-flight Mass Spectrometry (CE-TOF-MS) to investigate discriminatory metabolites from patients with oral cancer, pancreatic cancer, breast cancer, periodontal disease, and healthy controls, and identified 57 predictive metabolites of each individual disease. Most of these metabolites were present at relatively high concentrations in all three cancer patient groups compared with the periodontal disease patients and healthy subjects. In addition, three metabolites (taurine, piperidine, and a peak at 120.0801 m/z)

were oral cancer-specific markers with an AUC of 0.865, eight metabolites (leucine, isoleucine, tryptophan, valine, glutamic acid, phenylalanine, glutamine, and aspartic acid) were pancreatic cancer-specific markers with an AUC of 0.993. Using CE-TOF-MS as well, Tsuruoka et al. [57] demonstrated that two salivary metabolites (arginine and tyrosine) differed significantly between dementia patients and healthy subjects. Applying Ultrapformance Liquid Chromatography coupled with Quadrupole/Time-of-flight Mass Spectrometry (UPLC-QTOFMS), Wei et al. [58] found that a combination of three salivary metabolites (phenylalanine, valine, and lactic acid) could distinguish OSCC patients from healthy controls with high sensitivity and high specificity (86.5% and 82.4%, respectively) and Oral Leukoplakia (OLK) patients (94.6% and 84.4%, respectively).

### The microbiome

The oral cavity is a diverse habitat of bacteria and other microorganisms. A series of evidence shows that oral dysbiosis can lead to *Oral Diseases* such as periodontal diseases [59] and caries [60], as well as cancer and other systemic diseases [61-63]. In the past, it was thought that there were approximately 1000 bacterial species in the oral cavity [64]. Now, using Next-generation Sequencing (NGS) technology, the number of species could reach more than 10,000 [65]. The use of NGS, bacterial microarrays, and other emerging techniques can advance the investigation of the salivary microbiome and identify the association between special bacteria or other microorganisms and special oral or systemic diseases [66,67].

Mager et al. [68] used checkerboard DNA-DNA hybridization to evaluate the oral microbiota in saliva from patients with OSCC and healthy subjects and found a combination of three microbiotas (*Campylobacter* *gingivalis*, *Prevotella melaninogenica*, and *Streptococcus mitis*) that could be used as diagnostic biomarkers with 80% sensitivity and 82% specificity. Recently, using the Human Oral Microbe Identification Microarray (HOMIM) during the discovery phase and qPCR during the validation phase, Farrell et al. [69] profiled the salivary microbiota from patients with pancreatic cancer and healthy subjects; the results showed that 31 bacterial species were increased and 25 were decreased in pancreatic cancer patients compared with healthy subjects, and two bacterial candidates (*Neisseria elongate* and *Streptococcus mitis*) were able to distinguish patients with pancreatic cancer from healthy controls with 96.4% sensitivity and 82.1% specificity.

### The Mechanism of Salivary Diagnostics

Recent translational salivary biomarker development studies have supported that salivary biomarkers can discriminate oral and systemic disease patients from non-disease subjects [19,21,23,70,71]. However, the mechanisms of how diseases distal from the oral cavity would lead to the appearance of discriminatory biomarkers in saliva is largely unclear [72]. Investigating the origin of salivary biomarkers will be a significant goal in the development of salivary diagnostics, and the mechanisms of salivary diagnostics need to be elucidated.

Studies have increasingly demonstrated that some salivary

biomarkers might derive from systemic sources. Gao et al. [73] used mouse models of cancer to determine whether salivary biomarker profiles are affected by distal tumor development. Their data analysis of nerve growth factor production and the transcription factor Egr-1 suggests that the production of growth factors in tumor tissue represents one mechanism by which a distant tumor can alter the transcriptome of the salivary glands, and hence of saliva. While their report did not comprehensively demonstrate the mechanistic connection between systemic disease development and salivary biomarker alterations, it did begin to paint a picture of the concept that systemic networks exist in the human body that allow communication between distal diseases and the salivary glands. Signals transmitted through such networks might induce related signaling pathways that result in altered gene expression and protein translation, and thereby produce disease-induced salivary biomarker profiles. Therefore, the salivary transcriptomic profile might be composed of transcripts that originate in distant diseased tissues as well as transcripts that originate in salivary glands, and transcription factors that originate in distant tissues might alter the expression levels of these transcripts.

#### Exosomes: from formation to target

Cells continuously secrete a large number of microvesicles, macromolecular complexes, and small molecules into the extracellular space. Among the secreted microvesicles, nanoparticles called exosomes are currently undergoing intense scrutiny. Although exosomes were first discovered nearly 30 years ago, they were considered little more than cellular entities that acted to discard unwanted molecular components. However, over the past several years, evidence has begun to accumulate to suggest that these vesicles are signaling shuttles containing cell-specific collections of proteins, lipids, and genetic material that are transported to

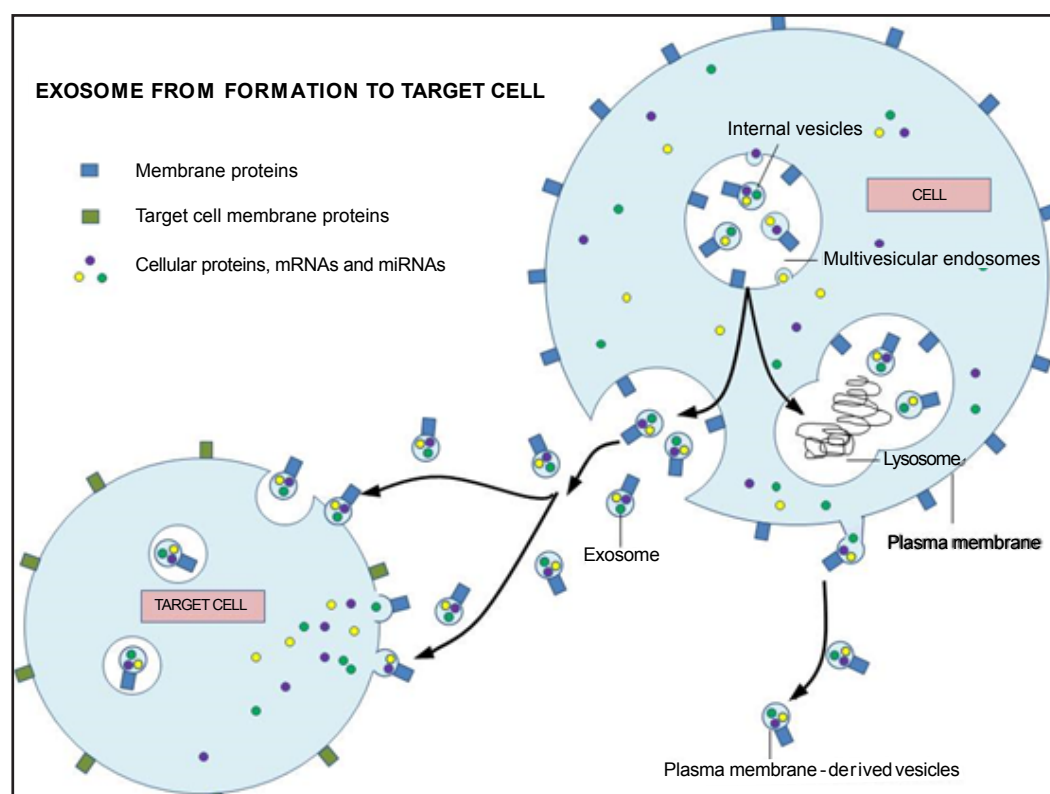
other cells, where they alter function and physiology. Hence, these findings have reignited interest in exosomes [74].

Exosomes are small vesicles (30-120 nm) that contain nucleic acid and protein, and they are perceived to carry this cargo between diverse locations in the body. They are distinguished in their genesis by being budded into endosomes to form Multivesicular Bodies (MVBs) in the cytoplasm. Exosomes are released to extracellular fluids by the fusion of these multivesicular bodies with the cell surface, resulting in secretion in bursts. They are secreted by most cell types [75-77], and are also found in abundance in body fluids, including blood, saliva, urine, and breast milk [78].

Specifically, at the beginning of exosome formation, internal vesicles are formed by the inward budding of cellular compartments known as Multivesicular Endosomes (MVEs). MVEs bud inward to form small internal vesicles that contain proteins, mRNA, and miRNA from the cytoplasm. When MVEs fuse with the cell membrane, these internal vesicles are released as exosomes, which can travel to distant tissues to influence various aspects of cellular behavior; alternatively, MVEs can fuse with lysosomes, which degrade MVE contents. Upon reaching their destinations (which are usually determined by the binding of specific ligands on their surfaces), exosomes enter the target cells in one of two ways: by being taken up by the target cell's endocytic pathway, or by fusing to the target cell's membrane and releasing their contents directly into the cytoplasm. Cells also secrete other membrane-derived vesicles, such as ectosomes, shed vesicles, or microvesicles, which bud directly from the cell's plasma membrane. These vesicles are also known to carry active proteins and RNAs, as well as some compounds that have never been described in exosomes before; however, little is known about their effects on distant tissues (*Figure 2*) [74].

#### Biological functions of exosomes

Exosomes have been proposed to signal both by binding to



*Figure 2. Exosome from formation to target cell.*

cell surface receptors through adhesion molecules [79] and by fusing with or being internalized by the recipient cell, potentially donating their own cytoplasm to the recipient cell [80,81]. The latter implies that exosomes may have mechanisms that are different from their function in the immune system. Lässer et al. recently discovered substantial amounts of RNA in exosomes derived from mast cells [79], which have the capacity to donate their RNA to other cells and can subsequently affect the protein production of a recipient cell. This finding suggests that RNA can be transferred between mammalian cells by an extracellular exosome-based transport mechanism, which has vast implications for the understanding of cell communication, regulation, and signaling, in addition to extensive therapeutic potential in many diseases.

Beyond their characteristic repertoire of surface markers, exosomes feature a wide range of surface and internal proteins specific to their source. When considering the diversity of cargo transported by exosomes, it should come as no surprise that exosomes have already been implicated in the development of polarized epithelial cells, neuronal development, and tumor growth [82]. Hunter et al. [83] identified the presence of various miRNAs in human serum exosomes. Because a single miRNA can regulate hundreds of genes and may act as a master regulator of processes, select subsets of miRNAs can be used as biomarkers of physiological and pathological states. Another feature that makes miRNAs excellent candidates for biomarker studies is their remarkable stability and resistance to degradation. In the clinical setting, exosomes are present in blood, saliva, plasma, urine, amniotic fluid, and effusions from malignant tumors. Given the relative ease and non-invasive *Nature* of isolating exosomes from patient samples, as well as their distinctive protein and nucleotide contents, several studies have suggested using exosomal biomarkers for disease diagnostic purposes. A study by Mi et al. showed that the expression of as few as two miRNAs can accurately discriminate acute lymphoid from acute myeloid leukemia [84], while Skog et al. [85] suggested that glioblastoma tumor-derived exosomes in patient serum carry a distinctive miRNA payload that can be used diagnostically. The majority of these studies investigated exosomes isolated from serum; only a few have focused on proteomic exosomal biomarkers in urine and saliva [86].

#### **The diagnostic and therapeutic potential of exosomes**

Recent research indicates that exosomes provide a mechanism for diagnostic and therapeutic salivary biomarkers that perform well in pancreatic cancer, breast cancer, and glioblastoma. Lau et al. [72] examined the hypothesis that pancreatic tumor-derived exosomes are mechanistically involved in the development of pancreatic cancer-discriminatory salivary transcriptomic biomarkers. They developed a pancreatic cancer mouse model that yielded discriminatory salivary biomarkers by implanting the mouse pancreatic cancer cell line Panc02 into the pancreas of the syngeneic C57BL/6 host. Then, they tested the role of pancreatic cancer-derived exosomes in the development of discriminatory salivary biomarkers by engineering a Panc02 cell line that is suppressed for exosome biogenesis, implanting it into the C56BL/6 mouse, and examining whether the discriminatory salivary biomarker

profile was ablated or disrupted. Suppression of exosome biogenesis resulted in the ablation of discriminatory salivary biomarker development. Their study supported the hypothesis that tumor-derived exosomes provide a mechanism for the development of discriminatory salivary biomarkers that are applicable to distal systemic diseases [87].

Lau et al. [72] also used an *In Vitro* breast cancer model to demonstrate that breast cancer-derived exosome-like microvesicles are capable of interacting with salivary gland cells, altering the composition of their secreted exosome-like microvesicles. They found that the salivary gland cells secrete exosome-like microvesicles that encapsulate both protein and mRNA. They also showed that the interaction with breast cancer-derived exosome-like microvesicles communicates with and activates the transcriptional machinery of the salivary gland cells. Thus, the interaction altered the composition of the salivary gland cell-derived exosome-like microvesicles on both the transcriptome and proteome levels.

### **Translational and Clinical Applications of Saliva Biomarkers**

As we gradually deepened our understanding of the mechanism of salivaomics and the advantages of saliva as a valuable diagnostic tool have been revealed, the surveillance of disease and general health has become a highly desirable goal. Evaluating alterations of salivary biomarkers can be applied to early detection, risk assessment, diagnosis, prognosis, and monitoring the progress of a variety of diseases, including cancers, infectious diseases, and immune diseases. Some oncogenic proteins that are detectable in saliva (e.g., HER-2) provide a basis for developing targeted therapy. Saliva also serves as a platform for personalized medicine. By comparing salivary biomarkers from patients receiving different treatments with different outcomes, saliva proteomics can also be used to monitor treatment response.

#### **Disease detection**

**Oral disease and oral cancer:** Salivary diagnostics have been developed to monitor periodontal disease and other *Oral Diseases*. Comparing the concentration of a select subset of salivary proteins (elastase, lactate dehydrogenase, IL-1 $\beta$ , IL-6, and tumor necrosis factor- $\alpha$ ) and the presence of five pathogens in patients with advanced periodontal disease and healthy controls, salivary IL-1 $\beta$  and multiple oral pathogens demonstrated an association with periodontitis [88]. And the validation study further proved that the combination of salivary MMP-8, IL-1 $\beta$ , and *Porphyromonas gingivalis* had a stronger association with moderate to severe periodontitis [89] (*Table 1*). A gingivitis-focused experiment that employed a multiplex protein array for selected biomarkers implicated in host defense, inflammation, tissue destruction, and angiogenesis has demonstrated that salivary biomarkers can also be used to evaluate the host response to bacterial invasion. Salivary IL-6 and IL-8 levels were shown to provide the best distinction between high and low responders [90]. In a study on dysplastic oral leukoplakia in relation to tobacco habits and periodontitis, increasing salivary IL-6 levels were also demonstrated to correlate with severity of dysplasia [91]. These findings indicate that salivary biomarkers have the potential to detect oral disease and determine disease stage.

**Table 1.** Discovered salivary biomarkers using epigenomics, transcriptomics, proteomics and Metabolomics for detecting oral disease, oral cancer, pancreas cancer, Lung Cancer, and breast cancer.

Disease	Approach		Markers
Periodontal disease	proteomic	ELISA [91]	IL-6
	Proteomic and microbial studies	ELISA [88,89]	IL-1 and <i>Aggregatibacter actinomycetemcomitans</i> , <i>Porphyromonas gingivalis</i> , <i>Prevotella intermedia</i> , <i>Tannerella forsythia</i> , and <i>Treponema denticola</i>
		Multiplex protein array discovery [90]	IL-6, IL-8
Oral cancer	Epigenomics	Candidate from previous study, Q-MSP discovery and validation [93]	KIF1A, EDNRB
	Transcriptomics	Microarray discovery and qPCR validation [20]	IL-8, SAT, IL-1B, OAZ1, H3F3A, DUSP, S100P
		Microarray discovery and qPCR validation [49]	IL8, IL1B, OAZ1, SAT
		Discovery and validation by RT-preamp-qPCR or candidate gene selection based on previous study, qRT-PCR quantification [16,94]	miR-200a, miR-125a and miR-31
		ELISA assessment and qPCR confirmation [95]	IL-8
Pancreatic cancer	Transcriptomics	Affymetrix array discovery and qRT-PCR validation [19]	KRAS, MBD3L2, ACRV1, DPM1
	Metabolomics	Discovery by CE-TOF-MS-based Metabolomics [56]	Leucine with isoleucine, tryptophan, valine, glutamic acid, phenylalanine, glutamine, aspartic acid
	Microbiome	microbial profiling using the Human Oral Microbe Identification Microarray [69]	<i>Neisseria elongata</i> and <i>Streptococcus mitis</i>
Lung Cancer	Transcriptomics	Microarray discovery and qRT-PCR verification and pre-validation [21]	CCNI, EGFR, FGF19, FRS2, GREB1
	Proteomics	Two-dimensional gel Electrophoresis and LCMS-MS [47]	Calprotectin, AZGP1, haptoglobin hp2
	Metabolomics	Discovery by SERS [105]	Unidentified peak wavelengths; 822, 884, 909, 925, 1009, 1,077, 1,369, 1,393, 1,721 cm-1
Breast cancer	Combination proteomic/transcriptomic approaches	Discovery by 2D-DIGE and RT-PCR/Affymetrix, validation by qRT-PCR[22]	mRNAs: CSTA, TPT1, IGF2BP1, GRM1, GRIK1, H6PD, MDM4, S100A8 Protein: CA6

Oral cancer (more than 90% of which are OSCC) is the sixth most common cancer worldwide, with an average 5-year survival rate of approximately 60% [92]. The key issue to reduce the mortality and morbidity associated with this disease is to develop strategies to identify OSCC at an early stage. Several biomarker candidates for OSCC have been reported, including Endothelin Receptor type B (EDNRB) hypermethylation [93], IL-8, and miRNAs such as miR-200a, miR-125a, and miR-31 [16,94,95], although only IL-8 proved to be a cost-efficient analyte for early OSCC detection [8]. Several biomarker panels have also been investigated for their ability to detect OSCC. Our prior salivary transcriptomic studies have discovered seven OSCC-associated salivary RNAs (IL-8, SAT, IL-1B, OAZ1, H3F3A, DUSP, and S100P). Initially, the levels of these RNAs were measured in a training set of 32 OSCC and 32 control samples using quantitative PCR, and a logistic regression model including four markers (IL-8, SAT, IL-1B, and OAZ1) achieved a cross-validation prediction accuracy rate of 81% showing their potential as biomarkers for OSCC detection [20]. The pre-validation study of these biomarkers demonstrated their feasibility regarding the discrimination of OSCC patients from control subjects [49]. Other proteomic, metabolomic, or epigenetic biomarkers have achieved lower specificity or have not been validated [10,56,58,96,97].

**Pancreatic cancer:** Pancreatic cancer is characterized by a propensity to rapidly disseminate to the lymphatic system and distant organs. Approximately 15% to 20% of patients have surgically resectable disease at the time of presentation; however, only approximately 20% of these patients survive to 5 years [98-100]. This aggressive biology, resistance to conventional and targeted therapeutic agents, and lack of biomarkers for early detection result in the poor outcomes of these patients. In a significant milestone, prospective sample

collection and a retrospective double-blinded validation study [19] showed that a salivary transcriptome profile could be used to detect early-stage resectable pancreatic cancer. Microbial profiling derived from the HOMIM was used to investigate variations in salivary microbiota between groups of 10 resectable patients with pancreatic cancer and 10 matched healthy controls. The profiling of bacterial candidates was further validated by qPCR on samples from an independent cohort of 28 resectable pancreatic cancer patients, 28 matched healthy controls, and 27 chronic pancreatitis patients, which yielded a ROC-plot AUC value of 0.90 (95% CI 0.78 to 0.96,  $p < 0.0001$ ) [69]. A metabolomic approach also identified pancreatic cancer-specific salivary metabolomic biomarkers that can distinguish pancreatic cancer from oral cancer, breast cancer, and cancer-free controls [56]. Both microbiomic and metabolomic biomarkers have achieved excellent accuracy for pancreatic cancer, although only the microbiomic panel has been validated.

**Lung Cancer:** Lung Cancer is the most common cause of cancer-related death in men and women in part because symptoms are frequently absent until the disease has already metastasized. Early detection represents a very promising approach to reducing Lung Cancer incidence and mortality. However, conventional diagnostic methods for Lung Cancer are unsuitable for widespread screening because they are expensive and occasionally miss tumors or invasive cancer [101,102]. Although computed tomography has been widely used for early Lung Cancer screening, it is associated with a high rate of false positives [103]. Biomarkers for Lung Cancer have the potential to improve early detection beyond the use of computed tomography scans [104]. With the 2D-DIGE proteomic analysis of saliva samples from Lung Cancer patients, 16 candidate biomarkers have been discovered and further verified [47]. Three candidate markers (calprotectin,

zinc- $\alpha$ -2-glycoprotein, and haptoglobin) achieved good sensitivity and excellent specificity and accuracy. And a transcriptomic biomarker profile including the B-Raf gene (BRAF, which is involved in directing cell growth), cyclin I (CCNI, which binds activated cyclin-dependent kinase 5), the epidermal growth factor receptor (EGFR), fibroblast growth factor 19 (FGF19), fibroblast growth factor receptor substrate 2 (FRS2), growth regulation by estrogen in breast cancer 1 (GREB1), and leucine zipper putative tumor suppressor 1 (LZTS1) has been identified, and a panel consisting of five of these markers is able to differentiate *Lung Cancer* patients from cancer-free subjects with 93.75% sensitivity and 82.81% specificity [21]. Surface-enhanced Raman spectroscopy (SERS) was recently applied to identify biomarkers for *Lung Cancer*. SERS revealed nine peaks (assigned to amino acids and nucleic acid bases) that are able to distinguish samples from patients with *Lung Cancer* and cancer-free controls with 86% accuracy, 94% sensitivity, and 81% specificity [105]. Therefore, both transcriptomic and proteomic approaches are proving highly useful in developing biomarkers for *Lung Cancer*.

**Breast cancer:** Breast cancer is the most common form of cancer and the second leading cause of cancer deaths in women in the United States [106]. Today, breast cancer detection depends on physical examination and imaging studies. Earlier investigation demonstrated the potential for salivary proteomic detection of breast cancer (e.g., salivary protein c-erbB-2) [44,107]. Recently, isobaric tags for relative and absolute quantitation (iTRAQ) technology combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been used to analyze saliva samples collected from 20 breast cancer patients and 10 healthy controls. Nine proteins were associated with breast cancer and exhibited 1.5-fold upregulation or downregulation [108]. The Affymetrix HG-U133-Plus-2.0 Array and 2D-DIGE were used to analyze the salivary transcriptomes and proteomes of 10 breast cancer patients and 10 matched controls during the discovery phase and 30 breast cancer patients and 63 controls during prevalidation [22]. Eight mRNA biomarkers and one protein biomarker were identified, yielding an accuracy of 92% (83% sensitive, 97% specific) on the preclinical validation sample set.

**Other systemic diseases:** In addition to providing powerful biomarkers to detect systemic cancers, saliva also provides biomarkers for autoimmune diseases, systemic microbial infections, and diabetes [8]. Although at significantly lower level than in serum, hepatitis C virus (HCV) RNA can be consistently detected in saliva from HCV-infected individuals using qPCR [109]. Label-free differential protein expression analysis using multidimensional LCMS-MS was conducted to characterize the proteome of saliva collected from type 2 diabetic patients compared with non-diabetic controls. Several proteins were found to have diagnostic potential for type 2 diabetes, but require additional study [110].

#### **Personalized medicine**

Cancer patients can be classified according to altered protein expression profiles, and statistical methodologies can be used subsequently to develop predictors for subgroups of patients that may benefit from targeted therapy. Trastuzumab is a humanized monoclonal antibody targeted against HER2, which is overexpressed in 25% to 30% of patients with breast

cancer. HER2 overexpression is an indicator for trastuzumab therapy. FDA-approved immunohistochemical and Fluorescence In situ Hybridization (FISH) methods [111] are now available to assess HER2 overexpression; however, these methods are only semi-quantitative and are interpreted in an operator-dependent manner. Because HER2 is detectable in human saliva, incorporation of this marker into clinical trials might assist in the classification of breast cancer patients and help determine which patient subgroups are most likely to benefit from such a molecular-targeted therapy. Saliva is also applicable for noninvasive prenatal diagnosis in predictive, preventive, and personalized medicine, especially for infants that are born prematurely. Amplification of salivary RNA and microarray analysis identified 9286 gene transcripts that exhibited statistically significant changes of expression across individuals over time [112]. The gene expression changes were closely linked to developmental pathways. A total of 2,186 genes were involved in successful oral feeding by Ingenuity Pathway Analysis [113]. The result elucidates the biological processes involved in oral feeding in the newborn at a molecular level, as well as novel pathways associated with successful oral feeding.

#### **Therapeutic efficiency**

Several candidate approaches have elucidated biomarkers for periodontitis and responses to therapy. In a study aiming to identify salivary biomarkers for chronic periodontitis, 33 participants received oral hygiene instructions alone and 35 participants received oral hygiene instructions in combination with conventional periodontal treatment comprising scaling and root planing. The levels of IL-1 $\beta$ , macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), matrix metalloproteinase-8 (MMP-8), and Osteoprotegerin (OPG) detected in saliva reflected disease severity and response to therapy [114].

#### **Saliva collection, processing, stabilizing and associated potential interference**

Saliva is an ideal non-invasive source of biomarker of systemic disease since the collection of whole saliva is noninvasive and easily accessible. The collection of individual salivary glands saliva is much more difficult and primarily useful for the detection of gland-specific pathology, i.e., infection and obstruction [115]. Glandular saliva specimens from both individual parotid glands and simultaneously from the submandibular/sublingual glands are collected by Lashley cups (placed over the orifices of Stenson's duct) and syringe aspiration (from the orifices of Wharton's duct located anteriorly in the floor of the mouth), respectively [116]. Whole saliva is easy to collect and is used for the evaluation of systemic disorders. Unstimulated saliva samples were collected between 9 a.m. and 10 a.m. with previously established protocols [117]. Subjects are asked to refrain from eating, drinking, smoking, or oral hygiene procedures for at least 1 hour before the collection. The clinical research procedure requires collection of whole saliva on ice followed by on-site centrifugation at 4°C. Centrifugation results in heterogeneous layers composed of supernatant, mucin, and cellular pellet. Saliva supernatant must be carefully removed by pipetting without disturbing the layers of cellular debris and mucin. Supernatant aliquots are stabilized by adding an RNase inhibitor or protein inhibitor cocktail [117]. Recently, direct

saliva transcriptome analysis (DSTA) of saliva supernatant has been demonstrated to be a suitable method for eliminating the need for mRNA isolation and RNA and protein aliquots remain stable at ambient temperature for several weeks [17,41]. While these procedures are not complicated, they still require the availability of specialized accessory equipment such as refrigerated centrifuge, ultra freezer, and pipettes that limit saliva sampling to clinical and research institutional sites with trained technician. Recently, we introduced iSCPSS (Integrated Saliva Collection, Processing, Stabilization and Storage) and demonstrated that iSCPSS yielded samples with similar protein and mRNA content and stability at ambient temperature compared to standard operating procedure [118]. The iSCPSS system consists of an absorbent collection pad attached to a lollipop-like handle equipped of compression tube, attachable bi-furcated filter unit, and collection tube. The iSCPSS system provides user-friendly systems for collecting, transporting and stabilizing saliva and reduces the need for trained personnel, specialized accessory equipment and specialized shipping. And the system will greatly increase the accessibility and affordability of saliva diagnostics to larger populations in remote settings instead of limiting in clinical and research institute.

### Challenges of Salivary Biomarker Discovery and Clinical Applications

Despite the fact that a series of salivary biomarkers have been reported to be valuable to detect oral and systemic diseases, there have been no standardization conditions and methods of saliva sample collection, processing, and storage prior to measurement. The differences in these factors among the different studies makes it difficult to compare the levels of the same biomarker reported between different labs and validate biomarkers reported in other labs. Then, highly accurate salivary biomarkers need to be discovered. In review of the reported potential salivary biomarkers, some of them were found to be valuable for detection in two different diseases: such as IL-1 $\beta$  and IL-8 in periodontal diseases [88,90] and oral cancer [20,49]. These findings suggest that the levels of some salivary constituents could be significantly changed by the presence of more than one type of disease, a fact which points to the necessity of validating the specificity of the reported potential salivary biomarkers with patients who have different diseases. Finally, most reported individual biomarkers have been only discovered without verification or validation, and they require further validation to confirm their performance before testing at the clinical level.

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### Conclusion

To be useful in a clinical setting, biomarkers should be accurate, reproducible, and collected using a non-invasive method that requires little preparation. Salivary diagnostics have these characteristics, and the rich biomarkers in saliva are applicable for detecting systemic disorders in addition to *Oral Diseases*. The current decision to use available diagnostic methods for many diseases is based on patients' symptoms and clinical information. The process of obtaining a final diagnosis can impose a burden on hospitals and a long waiting time for patients. As an accessible and noninvasive primary test for diseases, salivary diagnostics reduce the hospital's burden and the use of unnecessarily invasive procedures. As methods of stabilizing whole saliva are developed, salivary diagnostics can be performed correctly in the clinic without specially trained professionals, using point-of-care technology to allow the detection of disease without any preparation.

As detailed above, salivary diagnostics have both translational and clinical potentials. The development of high-throughput technology has revealed advanced insights toward an understanding of saliva as a reflection of the condition of the whole body. Exosomes provide a mechanism for the expression of diagnostic biomarkers in saliva, and also promote saliva as a powerful and unprecedented diagnostic tool in combination with high-throughput technology and bioinformatics platforms. The interpretation and utilization of this information will bolster the applicability of saliva to diagnosing disease, evaluating therapies, and designing personalized medicine.

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### Conflict of Interests

David Wong is co-founder of RNameTRIX Inc., a molecular diagnostic company. He holds equity in RNameTRIX, and serves as a company Director and Scientific Advisor. The University of California also holds equity in RNameTRIX. Intellectual property that David Wong invented and which was patented by the University of California has been licensed to RNameTRIX. Additionally, he is a paid consultant to PeriRx, GlaxoSmithKline and Wrigley.

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