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Salivary Biomarkers: Toward Future Clinical and Diagnostic Utilities

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SUMMARY

The pursuit of timely, cost-effective, accurate, and noninvasive diagnostic methodologies is an endeavor of urgency among clinicians and scientists alike. Detecting pathologies at their earliest stages can significantly affect patient discomfort, prognosis, therapeutic intervention, survival rates, and recurrence. Diagnosis and monitoring often require painful invasive procedures such as biopsies and repeated blood draws, adding undue stress to an already unpleasant experience. The discovery of saliva-based microbial, immunologic, and molecular biomarkers offers unique opportunities to bypass these measures by utilizing oral fluids to evaluate the condition of both healthy and diseased individuals. Here we discuss saliva and its significance as a source of indicators for local, systemic, and infectious disorders. We highlight contemporary innovations and explore recent discoveries that deem saliva a mediator of the body's physiological condition. Additionally, we examine the current state of salivary diagnostics and its associated technologies, future aspirations, and potential as the preferred route of disease detection, monitoring, and prognosis.

INTRODUCTION

The oral cavity is an intricate environment composed of multiple structures and tissues types working in concert. While each structure performs a unique function, all are colonized by bacteria and immersed in salivary fluids. Primarily considered an essential component of the digestive process, saliva serves to initiate the breakdown of lipids and starches via endogenous enzymes. However, in recent years, what we have come to understand about salivary secretions and the oral cavity has changed dramatically. Studies have shown that saliva actually contains a variety of molecular and microbial analytes (1, 2, 3, 4). Moreover, publications assert that these salivary constituents may actually be effective indicators of both local and systemic disorders (5). These revelations have formed the foundation of the field of salivary diagnostics and hence sparked investigations that culminated in the identification of saliva-based biomarkers for disorders ranging from cancer to infectious diseases (6, 7).

Although proteomic and transcriptomic indicators have yielded the most promising results to date (8, 9, 10, 11), information obtained from oral microbes and immunologic factors remains one of the more intriguing aspects in the pursuit of salivary biomarkers. Although the mechanism by which these disease indicators come to exist in saliva has not been explained fully, these findings insinuate that oral fluids may represent a significant source of discriminatory biomarkers for local, systemic, and infectious disorders.

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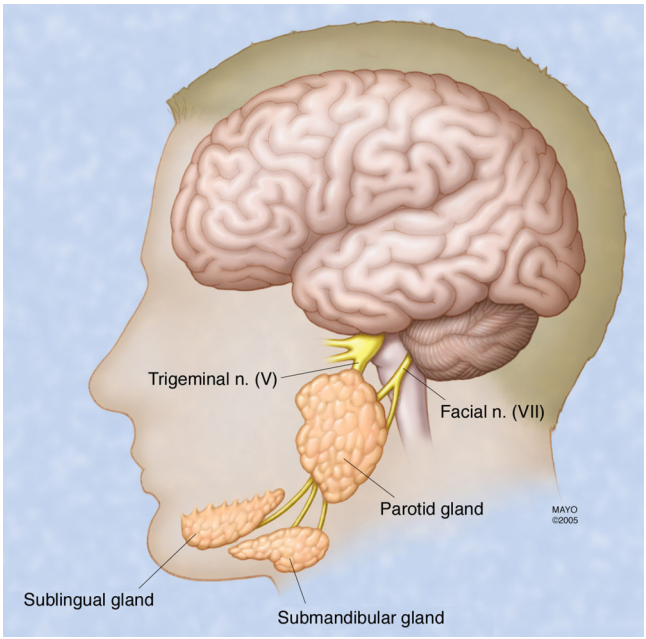

FIG 1 Locations of salivary glands (parotid, submandibular, and sublingual). The image shows a lateral view of the head showing the positions of the major salivary glands (the parotid, submandibular, and sublingual glands) and the nerves charged with their innervation (the trigeminal and facial nerves). Each gland is paired. The parotid glands are located just in front of each ear and are the largest of the three major salivary glands. The submandibular glands reside beneath the lower jaw just posterior and below the sublingual glands, which are located under the tongue. (Reprinted from reference 119 by permission of Mayo Foundation for Medical Education and Research.)

What was once deemed merely a digestive juice is now being considered a biological fluid capable of communicating an individual's current health status. Continued efforts in this field could lead to the establishment of clinically acceptable assays for the detection and monitoring of distinct disease states throughout the body. Here we review the utilization of saliva in molecular diagnostics and its potential future as a preferred mode of patient evaluation.

Properties of Saliva and the Salivary Glands

Human saliva is a clear, slightly acidic (pH 6.0 to 7.0) heterogeneous biofluid composed of water (99%), proteins (0.3%), and inorganic substances (0.2%) (12, 13, 14). On average, individual salivation can range from 0.3 to 0.7 ml of saliva per minute (15), producing a range of 1 to 1.5 liters daily. Saliva is multifunctional, serving not only to facilitate digestion, swallowing, tasting, and tissue lubrication but also as a protective barrier against pathogens.

Saliva is generated within the salivary glands by acinus cells, collected in small ducts, and subsequently released into the oral cavity (16). There are three major and numerous minor saliva-producing glands located in and around the mouth and throat (Fig. 1). Each gland is innervated autonomically, subject to parasympathetic and sympathetic stimulation, and considered to be exocrine in function. The three major glands, the parotid, submandibular, and sublingual glands, contribute >90% of total saliva, while the minor glands, the labial, buccal, lingual, and palatal glands, supply the remainder.

Each salivary gland is highly permeable and enveloped by capillaries (Fig. 2), a feature that allows for the free exchange of blood-based molecules into the adjacent saliva-producing acinus cells (17). Researchers postulate that blood-derived molecules entering salivary tissues via transcellular (e.g., passive and active transport) or paracellular (e.g., extracellular ultrafiltration) routes (18, 19, 20) could potentially influence the molecular constituency of oral fluids. This suggests that circulating biomarkers of disease absorbed by the salivary glands may possibly alter the biochemical composition of salivary secretions. Consequently, oral fluids may contain molecular information capable of communicating an individual's current state of health.

Although much remains to be proven, this hypothesized mechanism attempts to explain the etiology of saliva-based biomarkers. If this mechanism is true, it may be justifiable to consider oral fluids "a mirror of the body" or "a window on health status." Utilizing saliva as a physiological barometer could be the next step in molecular diagnostics. However, significant efforts must be undertaken and substantial milestones achieved in order to establish its efficacy over blood as the preferred diagnostic medium for disease detection.

Saliva versus Blood

Like saliva, blood is a complex bodily fluid known to contain a wide range of molecular components, including enzymes, hormones, antibodies, and growth factors (21, 22). While cells, tissues, stool, and other alternatives are routinely pursued, blood serum or plasma is traditionally and most frequently the source of measurable biomarkers. Although life-saving in many instances, the procedures required to collect and eventually analyze blood samples can often be expensive, problematic, and physically intrusive. Employing salivary fluids as a medium for biomarker development and evaluation alleviates subject/patient discomfort through the provision of a noninvasive method of disease detection.

Comparatively, saliva carries many advantages over blood, including the following:

1. Collection is undemanding. While blood sampling requires highly trained personnel, saliva procurement can be done by anyone, including self-collection.
2. The procedure is noninvasive. Sample procurement is painless, reducing the discomfort most individuals endure from biopsies and repeated blood draws, while encouraging others to participate in timely medical evaluations and screenings.
3. Samples are safer to handle. Salivary secretions contain factors that inhibit the infectivity of HIV, resulting in extremely low or negligible rates of oral transmission (23).
4. Samples are easier to ship and store. Saliva does not clot and requires less manipulation than blood.
5. The procedure is economical. Saliva is easily collected, shipped, and stored, resulting in decreased overall costs for patients and health care providers.

Despite these favorable attributes, the use of saliva as a diagnostic fluid has yet to become a mainstream idea. This partially stems from work revealing that while most analytes detected in the blood serum are also found in saliva, their levels are substantially diminished (24). For example, in healthy adults, IgA levels are normally

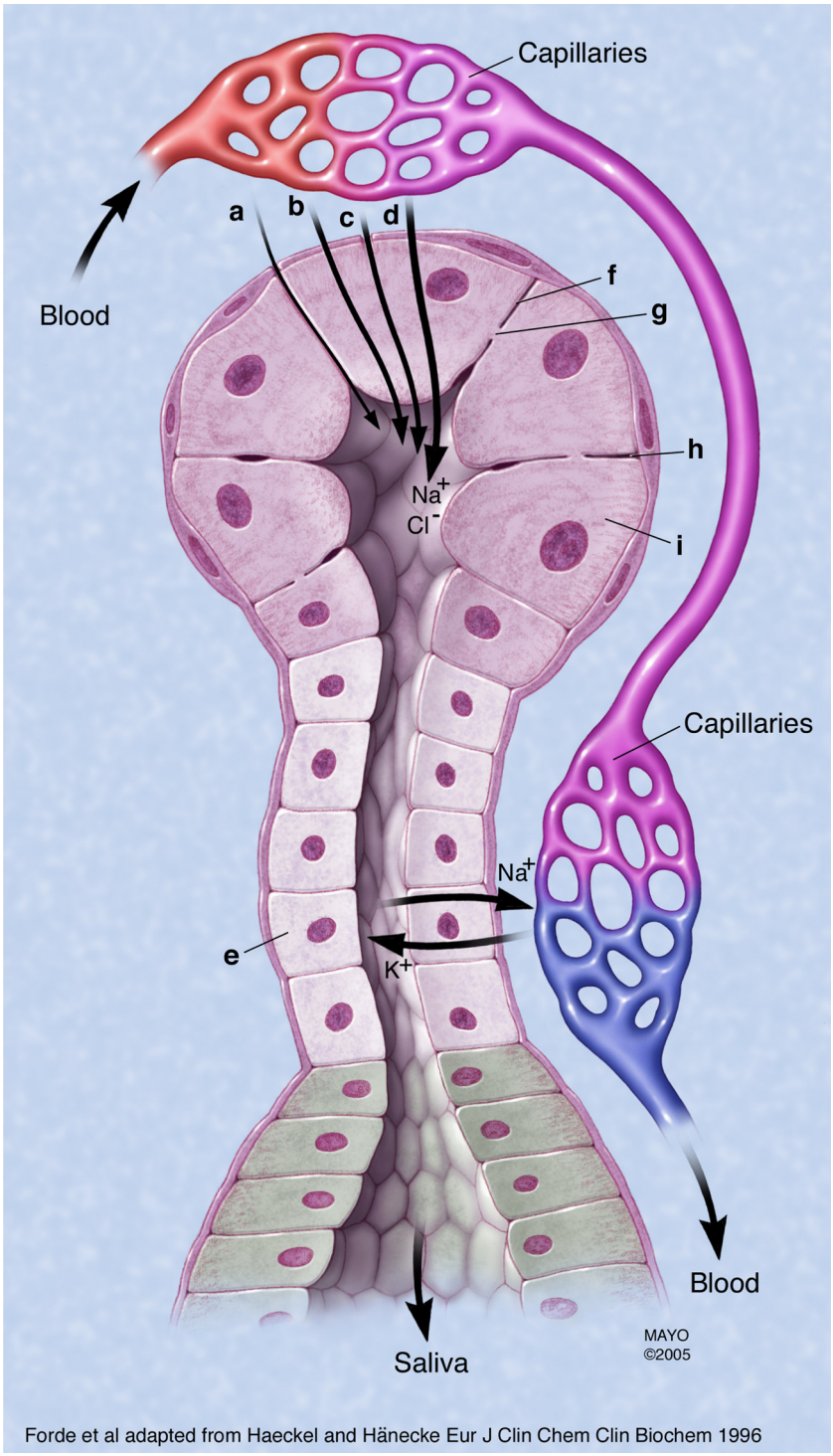

FIG 2 Mechanism of molecular transport from serum into salivary gland ducts. The image shows the proximity of a major salivary gland to the vascular system. Salivary glands are highly vascularized, allowing for the exchange of blood-based constituents. Acinus cells within the salivary glands absorb molecules from the blood and secrete salivary juices into the oral cavity. Alterations in the molecular composition of the blood may subsequently modify the composition of salivary secretions. Disease-specific blood-based biomarkers could sufficiently alter the output of salivary glands, yielding saliva-based biomarkers of systemic disorders. (Reprinted from reference 119 by permission of Mayo Foundation for Medical Education and Research. Adapted from reference 120.)

2.5 to 5 mg/ml in serum and 250 to 500 μg/ml in saliva. Similarly, IgG (5 to 30 mg/ml versus 5 to 30 μg/ml) and IgM (0.5 to 1 mg/ml versus 5 to 10 μg/ml) levels in serum are severalfold higher than those found in saliva (25). Even so, the correlation between salivary and blood-based constituents implies that while these two biofluids are separate and unique, they may be linked on a molecular level. Hence, it is imperative that we explore saliva as a potential alternative to blood- and tissue-based diagnostics.

TABLE 1 Common saliva collection devices

Company	Device
Salimetrics	Saliva collection aid
	Salimetrics oral swab
	Salimetrics children's swab
	Salimetrics infant's swab
Oasis Diagnostics	DNA·SAL
	UltraSal-2
	Super·SAL
Malvern Medical Developments	Oracol
DNA Genotek	ORAcollect · DNA
	Oragene · DNA
	Oragene · RNA
Immunalysis	Quantisal
Norgen	Saliva DNA collection and preservation device
Biomatrica	DNAgard

SALIVARY DIAGNOSTICS

Sample Collection

In order for a saliva-based diagnostic procedure to commence, one must first collect the necessary samples. Although simplistic in many ways, saliva collection can manifest unique issues within certain populations. These may include salivary flow rate, circadian rhythm, type of salivary gland, type of salivary stimulus, diet, age, physiological status, and method of collection. A Lashley cup, a noninvasive nickel-sized apparatus capable of accumulating fluids via suction, is occasionally employed to gather oral fluids from specific glands. Cotton swabs have also been used, but recent studies indicate that they may introduce unwanted bias (26). Additionally, a number of companies have introduced a variety of devices aimed at collecting saliva, the most common of which are summarized in Table 1.

Although draining, spitting, and suctioning remain the most common approaches (27), there is currently no universally accepted technique for sample collection, a fact that can hinder the research process by inhibiting the reliable reproduction of results. Having set guidelines standardizing the procedure could resolve any confounding issues between distant investigators and alleviate some of the inherent variability among individuals and populations. Regardless of the method used, it is imperative that subjects clean the oral cavity by rinsing with water to avoid the presence of contaminants prior to collection.

Biomarkers and Clinical Reality

According to the National Institutes of Health (NIH), a biomarker is an objectively measured and evaluated indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to therapeutic intervention (28). Summarily, biomarkers are entities within the body capable of providing impartial information regarding the current physiologic state of a living organism (29).

Biomarkers exist in a variety of different forms, including antibodies, microbes, DNA, RNA, lipids, metabolites, and proteins. Alterations in their concentration, structure, function, or action can be associated with the onset, progression, or even regression of a particular disorder or result from how the body responds to it (30). A collection of reliable and reproducible biomarkers unique to certain maladies is often referred to as a biomarker or molecular signature. Understanding and evaluating the significance of an individual's biomarker signature can be useful in determining the presence, location, and even likelihood of disease. Thus, biomarkers serve as a valuable and attractive tool in the detection, risk assessment, diagnosis, prognosis, and monitoring of disease (31).

The clinical realization of any biomarker or biomarker panel used for health risk assessment or prognosis is an arduous journey marked by extensive scrutiny. Potential biomarkers discovered in any of the "-omics" libraries (proteome, transcriptome, miRNAome [complement of microRNAs], metabolome, microbiome, or epigenome) are subjected to comprehensive evaluations, including preclinical and academic validations, prior to FDA evaluation and approval.

To minimize bias and reinforce significance, PRoBE (prospective specimen collection and retrospective blinded evaluation)-designed studies are typically employed. These protocols, which call for prospective sample collection and retrospective analysis prior to diagnosis, require large patient populations and the procurement and categorization of their samples and clinical information, respectively. Each sample is assessed via quantitative assay to determine the specificity, sensitivity, and reproducibility of the biomarker(s) in question. Further evaluations explore the capability of detecting and accurately measuring the markers in relatively low concentrations. Finally, in order for a biomarker to be used in a clinical assay, the following milestones must be achieved:

1. During preclinical testing, biomarkers must be developed using patient samples and confirmed at the *in vitro* or *in vivo* level.
2. During the feasibility analysis, biomarkers must be tested using small patient subpopulations to demonstrate their ability to discriminate diseased from healthy subjects.
3. During the validation process, biomarkers must be assayed accurately.
4. Statistical analysis must be done to evaluate the discriminatory accuracy of the biomarkers in a large patient population.
5. Subsequent to the reporting of a validated biomarker profile, efforts should be made to investigate their respective biochemical functions. Understanding the molecular roles of biomarkers could not only provide information concerning disease pathogenesis and progression but also support their position as evaluators of health (32).

ORAL DISEASE AND SALIVA

Microbial Biomarkers

As proposed by Haffajee and Socransky (33), there are 3 major points to take into account when determining the efficacy of microbial salivary diagnostics. First, in order for microbes to be considered disease-specific biomarkers, they must be associated directly with, but not necessarily the cause of, the condition in question. Next, if microbial biomarkers truly reflect health status, their regression or eradication should coincide with a positive therapeutic outcome. In other words, as a patient's condition im-

proves, the concentration or detectability of corresponding biomarkers should diminish.

The last consideration, and perhaps most meaningful, is whether microbial markers can be used to assess the risk of disease. If so, could a saliva-based microbial profile serve as a predictive indicator of disease, and is there a healthy profile to strive for? With regard to these issues, what is most exciting about oral microbial diagnostics is its potential utility beyond evaluating pathologies of the oral cavity. As discussed below, microbial and immunologic salivary profiles may be indicative not only of local disease but also of systemic maladies and infectious disorders.

The Oral Microbiome

The human oral cavity is a diverse habitat composed of teeth, gingival sulci, the tongue, hard and soft palates, the buccal mucosa, and tonsils. Each structure is colonized by bacteria and continuously bathed in saliva. Interestingly, studies have shown that salivary bacteria, including those shed from dental caries, may be surrogate indicators of disease useful in patient diagnosis, monitoring, and overall health evaluation (34, 35). With this in mind, a great deal of work has been done to define the human oral microbiome.

Established by the NIH, The Human Microbiome Project (36, 37) aimed to characterize the microbiological flora of several anatomical regions in healthy adult subjects, including the oral cavity. Even though certain studies report that 700 to 1,200 bacterial species (38, 39, 40, 41, 42) (www.homd.org) reside in the mouth, investigators using next-generation sequencing (NGS) suggest that this number could be as high as 10,000 (43, 44, 45, 46). While this is intriguing, further studies are required to clarify these numbers, as it is not clear whether such a large range of species truly colonize the oral cavity or are simply environmental transients.

Although most individuals harbor only 75 to 100 of the predominant bacterial species known to inhabit the oral cavity, 35% to 50% of those have yet to be cultivated. Ironically, recent analyses of sublingual plaque deposits indicate that many "uncultivable" specimens may actually be associated with oral health or disease (47, 48, 49). Fortunately, there are ancillary means by which to detect and monitor these and other species, using genomic analysis. The methodology describing this process has been reviewed in a number of recent articles (49, 50) and therefore is not discussed here.

Currently, most laboratory techniques, including NGS, bacterial microarrays, DNA hybridization, PCR, and quantitative PCR (qPCR), are employed in pursuit of specific questions as opposed to elucidating diagnostic values. Typically, the development of reliable disease markers follows the establishment of an association between specific bacterial species and specific diseases. Thus far, most studies utilizing the aforementioned methods have focused on certain oral sites, including subgingival plaque, tongue epithelial scrapings, and buccal mucosa, to determine the role of bacteria in oral health and disease. The following sections discuss early culture-based methods as well as contemporary molecular methods as they apply to salivary diagnostics and microbial biomarker development.

Early culture-based microbial diagnostics. Microbial salivary diagnostics is not a novel concept. Over 20 years ago, saliva-based tests were developed for *Streptococcus mutans* and *Lactobacillus* spp., two known etiological agents of dental caries. Dip slide tests for lactobacilli debuted in 1975 (51), followed by Cariescreen SM (52), an analysis that used agar-coated slides to detect and quantify salivary *S. mutans*. A similar test, called Dentocult SM Strip mutans, by Orion Diagnostica, quantifies *S. mutans* by incubating saliva-dipped test strips in selective broth media for 48 h (53). A software program called Cariogram evaluates the results, along with host dietary habits, plaque amount, and fluoride use, to calculate the relative risk of developing dental caries (54). Likewise, the caries risk test (55), a currently available diagnostic tool, simultaneously detects *S. mutans* and lactobacilli in saliva. This test, which has also been used to evaluate the relative risk of caries, utilizes blue mitis salivarius agar selective medium with bacitracin and Rogosa agar to detect *S. mutans* and lactobacilli, respectively (56, 57, 58). Although some studies have questioned their validity (59), these tests provide objective data used in clinical practice and research to detect bacteria and monitor health or disease status.

Molecular microbial diagnostics. As previously discussed, there is a clear rationale for using culture-based methods for risk assessment for dental caries. However, investigations drawing on culture-independent techniques are now producing evidence indicating the significance of molecular microbial analysis in identifying oral pathologies (60).

Recent studies employing quantitative 16S rRNA gene sequencing found several putative pathogens in the saliva of periodontitis patients in comparison to healthy controls (61, 62). Another investigation evaluating the synergy of microbial and molecular analyses found that biomarkers alone were insufficient discriminatory analytes (63), and only a combination of the microbial and molecular values could reasonably discern healthy from diseased subjects. Further studies have identified malodorous and caries-active subjects by using terminal restriction fragment length polymorphism (T-RFLP) analysis, deep sequencing, or human microbe identification microarrays (HOMIM), which are 16S rRNA-based microarrays capable of detecting 300 oral bacterial species, including those not yet cultivated (64, 65, 66).

SYSTEMIC DISEASE AND SALIVA

Infectious Disease

The diagnosis of systemic infectious diseases remains highly dependent upon the evaluation of blood and/or tissue samples. Although they are effective, these procedures are invasive and expensive and often require extensive time to obtain any meaningful diagnostic results. Furthermore, depending upon the practice setting, these types of tests may not be accessible for many patients and health care providers.

While saliva may serve to alleviate some of the challenges associated with more traditional diagnostic methodologies, it could also prove to be effective in terms of its accessibility. For example, patients with suspected HIV infections can now be screened for HIV-1 and -2 via a saliva-based enzyme-linked immunosorbent assay (ELISA) (67). Although positive results must be confirmed with a follow-up Western blot, this ELISA commonly generates accurate (99.3% sensitivity, 99.8% specificity) results rapidly (i.e., 20 min) and eliminates the necessity for invasive blood draws (68, 69). More importantly, though, it has now become widely accessible, as the FDA recently approved an over-the-counter, point-of-care ELISA kit, making HIV testing not only easy and increasingly available but also private (70). Currently, nearly 25% of all HIV-positive individuals are unaware of their infection (71). This specific population accounts for the majority of new HIV trans-

mission events annually (72). Providing the public with means to assess their HIV status could induce a reduction in the proportion of infected subjects by decreasing the likelihood of viral transmission. Furthermore, it may enhance long-term survival rates through facilitating the early initiation of antiretroviral therapy.

Although they are considerable, advances in saliva-based diagnoses are not unique to HIV infection. Significant progress has also been noted in the identification of additional systemic infections by way of oral fluids, including viral hepatitis, cytomegalovirus, malaria, and dengue fever. In fact, recent studies have revealed that antigens and/or antibodies for hepatitis A, B, and C viruses have been detected routinely in the salivary samples of infected individuals (6). Along with this, investigations have demonstrated the efficacy of hepatitis A virus (HAV) immunizations by assessing salivary IgG concentrations (73). Further work has focused on analyzing acute exposures to HAV by using a combination of saliva-based IgM, IgA, IgG, and HAV RNA tests (74). Acute exposure measurements, in particular, could prove to be a valuable clinical tool for the expeditious evaluation of large populations potentially exposed to contaminated food supplies. Utilizing saliva as a medium for point-of-care screenings and rapid detection could improve triage and early disease state management strategies by identifying the source of infection, thereby limiting further HAV exposures.

Unlike HAV, both hepatitis B virus (HBV) and hepatitis C virus (HCV) are commonly associated with chronic disease and can eventually result in severe liver-related complications such as cirrhosis and hepatocellular carcinoma. While each infection is fairly common, most patients are asymptomatic and remain unaware of their illness and the potential risk of disease progression and transmission. Traditional diagnosis and monitoring of HBV and HCV infections consist mainly of blood-based and serological tests evaluating viral load as well as viral antibodies and antigens. Interestingly, reports have indicated that HBV and HCV DNAs, antibodies, and viral antigens not only exist in the saliva of infected subjects but also correlate well with blood samples (75, 76, 77). These findings suggest a potential role for saliva as a noninvasive mode of HBV and HCV diagnosis and disease state monitoring.

Accordingly, a commercially available test that can rapidly identify HCV antibodies in saliva by using an enzyme immunoassay (EIA) was recently developed (78). Although the results obtained from this test draw parallels with those of serum immunoassays (97.5%), it has yet to obtain FDA approval and is currently not available in the United States. Even so, it is widely available in Europe and, if employed effectively, could possibly have a substantial impact on the early detection and management of HCV infections (79).

In consideration of the global health question, saliva-based diagnostics have been the primary focus of investigation for a variety of other infectious pathogens. Among these are several worldwide endemic microbes, including the malaria organism (*Plasmodium falciparum*), dengue virus, Ebola virus, and *Mycobacterium tuberculosis*, as well as a number of herpes simplex virus (HSV) family members, Epstein-Barr virus (EBV), cytomegalovirus (CMV), and human herpesvirus (HHV). For malaria, IgG antibodies directed against specific *Plasmodium falciparum* antigens can be detected in saliva and were found to correlate strongly with levels in plasma (80). Similarly, using antigen capture methods, IgA antibodies specific to dengue virus that correlate well with early secondary infection have been found in saliva (81). In contrast, *M. tuberculosis* and many viruses, including Ebola virus, HSV, EBV, HHV, and CMV, are most reliably detected directly using PCR methodologies (82, 83, 84, 85, 86, 87). Aside from CMV, where detection in the saliva of infants by PCR screening can identify newborns with congenital CMV infection, the clinical utility of saliva-based testing for most other pathogens has yet to be established definitively (87).

TABLE 2 Saliva-based biomarkers investigated for selected pathogens and their respective IVD statuses[a]

Pathogen(s)	Biomarker(s) investigated	IVD status	Reference(s)
HIV-1 and -2	IgG	Yes	67, 69, 70
Hepatitis A virus	IgM, IgA, IgG, and RNA	No	74
Hepatitis B virus	HbsAg, HbsAb, HbcAb, and DNA	No	75
Hepatitis C virus	IgG, RNA	No[b]	77, 78
Plasmodium falciparum	IgG	No	80
Dengue virus	IgA	No	81
Mycobacterium tuberculosis	DNA	No	82
Ebola virus	IgG, RNA, and antigen	No	83
Herpes simplex virus	DNA	No	84
Epstein-Barr virus	DNA	No	85
Human herpesvirus	DNA	No	86
Cytomegalovirus	DNA	Yes	87

[a] IVD, *in vitro* diagnostics.
[b] The Oraquick HCV rapid antibody test is not FDA approved but is commercially available and can be used for the qualitative detection of IgG antibodies to hepatitis C virus in oral fluid.

The global burden of both acute and chronic infectious diseases continues to increase. Reliable yet noninvasive and easily accessible diagnostic methods are not available for most infections, and as a result, many patients experience poor health outcomes. Saliva-based diagnostic methods could solve these challenges and have been established for certain infections, but continued work is necessary. Further efforts in this area should focus on the following: (i) optimizing point-of-care saliva-based testing for infection diagnosis, (ii) ensuring that testing methods remain noninvasive and provide reliable results rapidly, and (iii) improving accessibility for patients and providers while limiting health care costs. Table 2 summarizes the aforementioned diseases, their respective biomarkers, and their use in *in vitro* diagnostic tests.

Other Systemic Diseases

The employment of rapid, high-throughput NGS and 16S rRNA microarrays, including Phylochip (88) and Bactochip (89), has enabled us to better define the composition of the human oral microflora. Utilizing these and other technologies has allowed us to explore the oral microbiome and to evaluate its potential as an indicator of distal disorders. For instance, recent studies found that pediatric Crohn's patients presented with a statistically significant decrease in overall oral microbiological diversity (90). Further reports state that individuals with conditions defined by aberrant oral bacterial growth are at increased risk for pancreatic cancer (91, 92, 93). This potential correlation between oral microbes and peripheral disease prompted us to examine the salivary microbiome in an effort to identify markers of pancreatic cancer. Using HOMIM, we evaluated the oral microbiota of individuals diagnosed with either pancreatic cancer or chronic pancreatitis

TABLE 3 Saliva-based biomarkers of selected oral and systemic diseases

Disease	Oral microbial biomarkers investigated	Reference(s)
Oral cancer	*Capnocytophaga gingivalis*, *Prevotella melaninogenica*, *Streptococcus mitis*	7
Crohn's disease	*Fusobacteria*, *Firmicutes*	90
Pancreatic cancer	*Neisseria elongata*, *Streptococcus mitis*	94
Chronic pancreatitis	*Granulicatella adiacens*, *Streptococcus mitis*	94
Periodontal disease	*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Campylobacter rectus*, *Treponema denticola*	34, 49, 61, 62
Dental caries	*Streptococcus mutans*, *Lactobacillus* spp.	51, 52
Obesity	*Selenomonas noxia*	118

(94). In summary, our efforts revealed an increase in 31 and a decrease in 25 bacterial species/clusters in the saliva of pancreatic cancer patients versus healthy controls ($n = 10$ each). Two oral bacterial candidates (*Neisseria elongata* and *Streptococcus mitis*) were validated in an independent sample population and showed significant reductions ($P < 0.05$; qPCR) in pancreatic cancer subjects compared to controls ($n = 56$ total). Synergistically, these microbial biomarkers yielded an area under the concentration-time curve (AUC) of 0.90 (95% confidence interval [95% CI], 0.78 to 0.96; $P < 0.0001$) for a receiver operating characteristic (ROC) plot, with 96.4% sensitivity and 82.1% specificity in distinguishing pancreatic cancer patients from healthy subjects. Additional analyses returned significant differences ($P < 0.05$; qPCR) in the concentrations of *Granulicatella adiacens* and *Streptococcus mitis* in the oral cavity between chronic pancreatitis and control samples ($n = 55$ total).

To our knowledge, this was the first study illustrating the association between the salivary microbiome and pancreatic pathologies. However, inquiries of this nature are not uncommon. As overviewed in Table 3, saliva-based microbial biomarkers have also been linked to oral cancer and even obesity.

Suggesting that oral microbes may function as indicators of systemic disease, the aforementioned investigation describes the elucidation of saliva-based microbiological markers for pancreatic cancer. Although the data are substantial, what must be established now is how these indicators come to exist in the oral cavity and whether the oral microflora is an accurate identifier of additional systemic conditions. Understanding how alterations in the oral microbiome relate to local and systemic disorders may provide critical input regarding disease pathogenesis, diagnosis, monitoring, and prognosis. Establishing disease-specific microbiological signatures could lead to the development of simple tests targeting discriminatory microbes capable of identifying particular pathologies. Early detection, especially in high-risk populations, will allow for more expeditious therapeutic interventions that may inhibit the progression, or even the onset, of pancreatic cancer and other disorders, leading to more positive outcomes.

OTHER BIOMARKERS IN SALIVA

Transcriptomics

As stated above, studies have shown that salivary secretions not only harbor RNA molecules but also may be a highly promising source of discriminatory biomarkers. To that end, recent investigations have identified more than 3,000 species of mRNA and over 300 miRNAs in the salivary fluids of healthy and diseased subjects, suggesting the possibility that transcriptomic analysis may yield valuable information regarding the condition of the body (95). With this in mind, a number of investigations have reported the identification of salivary biomarkers for Sjogren's syndrome and a number of cancers (95, 96, 97, 98). While further analyses need to be performed, these outcomes suggest a substantial role for the salivary transcriptome as a viable and noninvasive source of disease-specific biomarkers.

Proteomics

Human saliva contains a large collection of diverse proteins, each with distinct biological functions. While some aid in digestion and lubricating oral cavity, others help to maintain homeostasis and oppose pathogenic bacteria. Although its proteomic content is estimated to be only 30% that of blood (99), saliva is actively being investigated as a rich source of protein biomarkers (100) capable of discerning healthy from diseased subjects (101). To that end, numerous studies have revealed discriminatory protein profiles for oral cancer, diabetes, periodontal disease, AIDS, and mammary gland carcinoma (102, 103, 104, 105, 106, 107).

Methylomics

Known to affect mammalian development, cellular differentiation, and carcinogenesis, DNA methylation induces cells to maintain or alter unique characteristics by controlling and modulating gene expression (108, 109, 110). Curiously, several investigations are now reporting saliva-based genomic methylation analyses discerning oral squamous cell carcinoma (OSCC) (111) and head and neck squamous cell carcinoma (HNSCC) patients from their respective controls (112, 113). Additionally, a number of studies have explored local and global epigenetic alterations with regard to age (114, 115, 116), suggesting the possibility of saliva-based predictive screenings for age-related diseases.

Another interesting aspect of salivary methylomics is its potential role in forensic science and body fluid identification. As evidenced in a recent study, 5 tissue-specific differentially methylated regions (tDMRs) were distinguished via bisulfite sequencing using pooled DNA from blood, saliva, semen, menstrual blood, and vaginal fluid (117). Though preliminary, these results are promising and lay the groundwork for future genomewide DNA methylation analyses. Future applications may include the use of this technology as a standard forensic technique in the determination of unknown host bodily fluids.

CONCLUSION

Accurate and reliable early-stage disease detection combined with noninvasive modes of sample collection is the holy grail of molecular diagnostics. Saliva is a biofluid potentially rich in diagnostic indicators for both oral and systemic disorders. In recent years, numerous methodologies have emerged for evaluating the microbial and molecular constituency of saliva. As detailed above, unique saliva-based biomarker profiles can be correlated to cer-

tain diseases and may provide critical information regarding an individual's current physiologic state. Discovering, validating, and understanding saliva-based biomarkers could have a considerable role in establishing oral fluids as a credible diagnostic biofluid.

The seminal field of salivary diagnostics indeed has great translational and clinical potentials. Continuing advancements in high-throughput technologies have propelled this genre by revealing unprecedented insights toward understanding the salivary milieu as a reflection of the body's overall health. Correct interpretation and utilization of this information may be useful not only for identifying local and systemic disorders but also to aid in the design and modification of therapies.

One objective shared among researchers and clinicians alike is to noninvasively assess and monitor the physiological status of healthy and diseased individuals. The exploration and establishment of saliva as a diagnostic tool may fulfill this objective by providing a safe and effective means by which to evaluate patients and personalize their treatment.

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