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Developments in diagnostic applications of saliva in oral and systemic diseases- A comprehensive review

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Abstract

Human saliva is not just the fluid in our mouth, but it mirrors our body's health and well being. Biomolecules that are circulating in the blood are also found in human saliva. It consists of approximately about 2,000 proteins, and most significantly, 26% of these proteins are also found in blood, therefore emphasizes the saliva's importance as an added biological resource for disease diagnosis and monitoring, as well as an ultimate diagnostic medium to establish a person's response to treatment. The field of saliva diagnostics (SDs) began in the early 60s when salivary calcium levels were found to be elevated in cystic fibrosis patients, and 50 years on now how the field has unmitigated to an unpredicted distance due to the development of increasingly sensitive detection techniques. Hence, today in the era of nanotechnology and genomics, field of salivary diagnostics is promising a dramatic change in disease diagnosis and clinical monitoring. It has expanded into detection of cancer, heart and infectious diseases. Today we are using human saliva to detect illicit drugs, alcohol, to measure hormone levels, especially estrogen levels in women suffering from hormone imbalance, endometriosis, and to diagnose HIV virus in patients suspected of having AIDS. In addition, there are home-based saliva tests that one can order over the Internet to test one's own cholesterol levels and also can verify the risk of developing prostate cancer. With the development of novel, more sensitive detection technology platforms, and the innovation of standardized analytical tools, establishment of reference intervals will make saliva diagnostic a reality in the near future. Especially in the areas of population-based screening programs, confirmatory diagnostics, risk stratification, forensic and therapy response monitoring. So, it represents a progressively more valuable complementary means of diagnosis.

Keywords: Saliva, Salivary diagnostics.

Introduction

The ability to monitor health status, disease onset, progression, and treatment outcome through noninvasive means is a highly desirable goal in health care promotion and delivery. Saliva is a perfect medium to be explored for health and disease surveillance.[1-3] The use of human saliva as a diagnostic and prognostic fluid has until recently been somewhat disregarded. Recently, several proteomics studies contributed to the partial elucidation of the salivary proteome (more than 2400 protein components have been characterized) both in terms of composition, contributions to whole saliva and genetic/physiological variability. On this basis, is not too optimistic to believe that in the near future, human saliva could become a relevant diagnostic fluid.[4] In this review, the characterization of proteomic, genomic approaches of new salivary markers

in the diagnosis of various cancers, oral and systemic diseases and advantages of transcriptome analysis is discussed.

Saliva

Saliva, science has revealed, is much more than water. It is packed with proteins that help control the teeming hordes of microbes in our mouths. It is stuffed with substances that make us spit gristly, stop our teeth from dissolving and help heal wounds. It is packed with a plethora of hormones and other chemicals revealing anything from whether one smokes to whether one is stressed.

Saliva is a clear, slightly acidic mucoserous exocrine Biofluid produced in the oral cavity by three major (parotid, submandibular and sublingual) and around 450-750 minor salivary glands (situated on the tongue, buccal mucosa and palate except anterior part of the hard palate and gums). Whole saliva (WS) is a mixture of the secretions of the major and minor salivary glands, mucosal transudations, gingival crevicular fluid, serum and blood derivatives from oral wounds, desquamated epithelial cells, expectorated bronchial and nasal secretions, bacteria and bacterial products, viruses and fungi, other cellular components, and food debris. It is a complex fluid containing an entire library of hormones, proteins, enzymes, antibodies, antimicrobial constituents, and cytokines.[5] The mechanism of entry of these constituents of the blood into the saliva is by transcellular, passive intracellular diffusion and active transport, or paracellular routes by extracellular ultrafiltration within the salivary glands or through the gingival crevice.[6,7]

The significance of Saliva to be used as a diagnostic fluid

Saliva as a diagnostic fluid offers a distinctive advantages over serum because it can be collected non-invasively and does not require special equipment for collection and storage as unlike blood, saliva does not clot. Advantageous for people in whom blood drawing is difficult as in obese, hemophiliacs and patients who are fearful of prick. WS can be used for diagnosis of systemic diseases, because it contains serum constituents, for some diagnostic purposes, salivary biomarkers proved more useful than serum analysis.[8,9] These constituents are derived from the local vasculature of the salivary glands and gingival crevicular fluid.[8] Many advantages of saliva as a clinical tool over serum and tissues are a noninvasive collection of sample, smaller sample aliquots, good cooperation with patients, cost effectiveness, easy storage and transportation, repeated sampling for monitoring over time, greater sensitivity, and correlation with levels in blood.[10]

Most often cited criticism of using saliva as a diagnostic fluid is these biomarkers are present in amounts that are too low to be detected reliably. However, this is no longer a limitation due to the development of increasingly sensitive detection techniques. Hence today in the era of nanotechnology and genomics, field of SDs is promising a dramatic change in disease diagnosis and clinical monitoring. With current research, the gap is closing rapidly between the use of saliva and other biofluids for disease diagnostics.[2] The emerging field of microbiology and nanotechnology based biosensor will overcome the detection barriers.

Two prerequisites exist before the goal of SDs can be achieved: identification of specific biomarkers associated with a health or disease state and the development of technologies that can discriminate between the bio makers. A recent initiative of the National Institute of Dental and Craniofacial Research (NIDCR) has created a roadmap to achieve these goals through the use of oral fluids as the diagnostic medium to scrutinize the health and/or disease status of patients. This is an ideal opportunity to optimize state-of-the-art saliva-based biosensors for salivary biomarkers that discriminate between diseases.[10]

This is an exciting time, as we are seeing the applications of SDs for oral diseases, which will be followed soon by the application to high-impact systemic diseases, using highly informative panels of salivary proteomic and genomic biomarkers. This will enable researchers to bridge oral health research with systemic disease diagnostics via a biofluid that filters, processes and secretes itself from the vasculature that nourishes the salivary glands into the oral cavity.[10]

Salivary analysis can be done for the diagnosis of the following conditions[8]

1. Hereditary disease
2. Autoimmune disease
3. Malignancy
4. Infection
5. Monitoring of levels of hormones
6. Monitoring of levels of drugs
7. Bone turnover marker in saliva
8. Forensic Evidence
9. Oral diseases
10. Diagnosis of Oral Disease with Relevance for Systemic Diseases

1. Hereditary diseases

Cystic fibrosis (CF) is a genetically transmitted disease of children and young adults, which is considered a generalized exocrinopathy. A defective electrolyte transport in epithelial cells and viscous mucus secretions from glands and epithelia characterize this disorder. The organs most affected in CF are: sweat glands, the lungs and the pancreas. Elevations in electrolytes (sodium, chloride, calcium, and phosphorus), urea and uric acid, total protein and lipids were observed in the submandibuar saliva of CF patients.[11]

21-Hydroxylase deficiency is an inherited disorder of steroidogenesis which leads to congenital adrenal hyperplasia. Early morning salivary levels of 17-hydroxyprogesterone (17-OHP) determined by ELISA is an excellent screening test for the diagnosis of non-classic 21-hydroxylase deficiency, since the salivary levels accurately reflected serum levels of 17-OHP.[12]

2. Autoimmune diseases

Sjogrens syndrome (SS), a chronic autoimmune disease, characterized by dysfunction of salivary and lacrimal glands, keratoconjunctivitis sicca, xerostomia, in addition to serological abnormalities. Researchers have measured specific concentrations of cytokines in the saliva of patients with SS for their eventual use in diagnosis. Interleukins 2 and 6 are found in levels significantly high in individuals that suffer from this disease, thus, SDs can be useful in the diagnosis of SS.[13]

Multiple Sclerosis (MS) is an inflammatory disease characterized by loss of myelin and scarring caused due to destruction/failure of myelin producing cells by the immune system. SDs shows no significant change in the saliva of patients with multiple sclerosis except for a reduction in IgA production.[14]

Sarcoidosis, is an inflammatory disease of the lymph nodes, lungs, liver, eyes, skin, or other tissues. SDs demonstrates a decrease in the secretion volume of saliva in addition to a reduction in the enzyme activity of alpha-amylase and kallikrin in most of these patients.

3. Malignancy

Head and neck cancer, SDs may aid in the early detection and screening of certain malignant tumors and aids in monitoring the efficacy of treatment. The mRNA levels of specific proteins are elevated in the saliva of head and neck cancer patients.

p53 is a tumor suppressor protein which is produced in cells exposed to various types of DNA-damaging stress. Inactivation of this suppressor through mutation is considered a frequent occurrence in the development of human cancer. Accumulation of inactive p53 protein occurs, which in turn lead to the production of antibodies directed against this p53 protein. The p53 antibodies can be detected in the saliva of patients diagnosed with oral squamous cell carcinoma (OSCC), and can thus assist in the early detection and screening. High-positive correlation was observed between salivary defensin-1 levels and serum levels of OSCC related antigen. Higher levels of salivary nitrate and nitrite, and increased activity of nitrate reductase, were found in oral cancer patients compared with healthy individuals.[15]

Breast cancer, elevated levels of tumor markers c-erbB-2 (erb) and cancer antigen 15-3 (CA15-3) were found in the saliva of women diagnosed with breast cancer, as compared with patients with benign lesions and healthy controls. So they hold greater promise for the early screening and detection of breast cancer.[16] CA-125 is a tumor marker for cancer, elevated salivary levels of CA-125 were detected in patients with untreated breast cancer than healthy controls and patients who were treated for breast cancer. A positive correlation was found between salivary and serum levels of CA-125.[17]

4. Infectious diseases

Saliva contains immunoglobulins (IgA, IgM, IgG) that originate from two sources: the salivary glands and serum. Antibodies against viruses, bacteria, fungal and parasite can be detected in saliva and can aid in the diagnosis of infections

Bacterial infections

Helicobacter pylori infection has been associated with peptic ulcer and chronic gastritis. Oral cavity may be the source of infection. There was considerable variation in the detection rate of *H. pylori* DNA in salivary samples.

Shigella, children infected with Shigella revealed higher titers of anti-Shiga toxin antibody in comparison with healthy controls.[18]

Pneumococcal pneumonia the detection of pneumococcal C polysaccharide in saliva by ELISA offers a valuable complement to conventional diagnostic methods.[19]

Lyme disease caused by the spirochete Borrelia burgdorferi and transmits to humans by blood feeding

ticks. The detection of anti-tick antibody in saliva serves as a screening mechanism for individuals at risk for Lyme disease.[20]

Taenia solium specific antibody to Taenia solium larvae in serum demonstrated greater sensitivity than antibody in saliva for identification of neurocysticercosis.[21]

E. histolytica causative agent for ameobic liver abscess is challenging to diagnose, but DNA in saliva by real time PCR assay could be used for diagnosis.[22]

Viral infections

HIV, antibody to HIV in WS of infected individuals was detected by ELISA and Western blot assay, correlated with serum antibody levels. Salivary IgA levels to HIV decline as infected patients become symptomatic. It was suggested that detection of IgA antibody to HIV in saliva may, therefore, be a prognostic indicator of the progression of HIV infection. Several salivary and oral fluid tests have been developed for HIV diagnosis. Orasure is the only FDA- approved, commercially available testing system. It detects antibodies against the p24 antigen of HIV.[23, 24]

Acute hepatitis A (HAV) and hepatitis B (HBV) were diagnosed based on the presence of IgM antibodies in saliva. Quantitative detection of DNA is used to evaluate the level of virus in the body and also been used for screening hepatitis B surface antigen (HbsAg) in epidemiological studies.[25, 26]

Rotavirus for newborn infants, the salivary IgA response was found to be a better marker of rotavirus infection than the serum antibody response.[27]

Herpes simplex virus type-1 (HSV-1) reactivation is involved in the pathogenesis of Bell's palsy and PCR based identification of virus DNA in saliva is a useful method for the early detection of HSV-1 reactivation.[28]

Dengue is a mosquito-transmitted viral disease. Salivary anti-dengue IgM and IgG demonstrated sensitivity of 92% and specificity of 100% in the diagnosis of infection.[29] Apart from this even Measles, Mumps, and Rubella can also be diagnosed by using SDs.[30]

Fungal infections

The salivary fungal count analysis provides valuable information in cases of oral candidiasis, the alterations in the salivary proteins, like immunoglobulins, Hsp70, calprotectin, histatins, mucins, basic proline rich proteins and peroxidases also have important diagnostic value in these cases.[14]

5. The Monitoring of Hormone Levels

Cortisol due to their lipid solubility, steroid hormones can be detected in saliva. It was found to be useful in identifying patients with Cushing's syndrome, Addison's disease and the effect of stress.[31]

Aldosterone, recent evidence demonstrates an increased incidence of primary aldosteronism in approximately 10% of the hypertensive population, making noninvasive and simple screening methods necessary. Salivary aldosterone correlated significantly to plasma aldosterone levels ($r = 0.60$), and increased aldosterone levels were found in both serum and saliva of patients with primary aldosteronism (Conn's syndrome).[32]

Testosterone and dehydroepiandrosterone, salivary concentrations were found to be 1.5-7.5% of the serum concentrations of these hormones. Monitoring salivary testosterone levels may be useful to assess testicular function and behavioral studies of aggression, depression, abuse, violent and antisocial behavior.

Progesterone, salivary progesterone levels showed good correlation with free serum levels, elevated salivary estriol is associated with increased risk of preterm birth.[33]

Insulin: A positive correlation between saliva and serum insulin levels following a glucose tolerance test was reported for healthy and diabetic patients.[34] Saliva also contains multiple components whose concentrations are altered by diabetes, some of which (glucose, α-amylase, and ghrelin) have strong diagnostic potential.[35]

6. Detection of drugs

A fundamental prerequisite for diagnostic application of saliva is a definable relationship between the concentration of a therapeutic drug in blood and saliva. Only the unbound fraction of the drug in serum is available for diffusion into saliva, this unbound drug is usually the pharmacologically active fraction. This may represent an advantage of drug monitoring in saliva in comparison with serum, where both bound and unbound fractions of a drug can be detected.

Saliva has been widely studied as a medium for pharmacokinetics and therapeutic drug monitoring.[36, 37] In recent years, there has been vast interest from law enforcement agencies to develop oral fluid based point of

detection methods for illegal drugs and/or legal intoxication limits, resulting in an international cooperative study for roadside test [e.g.,European Commission on Roadside testing, assessment (Rosita). In this study, a large number of recreational and illicit drugs (e.g.,amphetamine, opium, alcohol, lysergic acid diethylamide, marijuana, and phencyclidine, etc.) and their metabolites have been evaluated in saliva samples in comparison to their serum counterparts).[38-40]

Saliva may be used for monitoring patient compliance with psychiatric medications and useful for monitoring anti-epileptic and anti-cancer drugs. Estimation of salivary carbamazepine levels is a predictable and convenient method of drug monitoring in epileptic patient, and a positive correlation (r = 0.659) between salivary and serum carbamazepine levels was observed.[41]

Ethanol is unionized in serum, is not protein-bound, and has a low molecular weight and lipid solubility, as a result it diffuses rapidly into saliva. The saliva sample should be obtained at least 20min following ingestion. Other recreational drugs that can be identified in saliva are amphetamines, barbiturates, benzodiazepines, cocaine, phencyclidine, and opioids.[41]

Nicotine, saliva helps to monitor tobacco smoking and exposure to tobacco smoke. The major nicotine metabolite cotinine is tobacco-specific and has a relatively long half-life (17hours) compared with nicotine. It was investigated as an indicator of exposure to tobacco in active and passive smoking, useful in monitoring compliance with smoking cessation programs.[42]

7. Bone Turnover Marker in Saliva

Mcgehee and Johnson used commercially available ELISA to test for the presence of osteocalcin (OC) and pyridinoline (PYD) in the whole human saliva of women. Level of OC and PYD in saliva correlated reasonably well with calcaneus bone mineral density BMD/t scores.[43]

8. Forensic Evidence

Saliva may be found on victims of several violent crimes, aberrant genetic material (deoxyribonucleic acid; DNA) and the messenger ribonucleic acid (mRNA) that helps process the genetic information into a protein from cells can also be detected in saliva. It can potentially be recovered from bite marks, cigarette butts, postage stamps, envelopes and other objects. During the biting process, saliva is deposited on the skin or the object surface in enough amount to allow typing of the DNA. PCR allows replication of thousands of copies of a specific DNA sequence in vitro, enabling the study of small amounts of DNA.[44]

8. Oral diseases

Dental caries, saliva secretion rate and buffering capacity have proven to be sensitive parameters in caries prediction models. High numbers of *S. mutans* and *Lactobacillus* indicate a shift in oral microflora from healthy to more cariogenic. Diagnostic kits for *S. mutans* and *Lactobacillus* counting and salivary buffering capacity widely use in dental practice and can be conducted without laboratory facilities.

Periodontal Disease (PD), another oral disease, for which salivary diagnostics are evaluated, Mutations in the cathepsin-C gene has been identified as causal for the Papillon-Lefèvre syndrome. People at high risk for PD can be determined by genetic screening. DNA can easily be isolated from oral epithelial cells, collected by use of a buccal swab. The loss of attachment and deepening of the periodontal pocket leads to increased leakage of a serum-like fluid designated gingival crevicular fluid, into the oral cavity. During active periods of the disease increased levels of inflammatory markers, like interleukins are demonstrated in saliva.

Several bacteria have been associated with PD, therefore, prior to antibiotic treatment pathogens are determined by culturing or PCR techniques. Nevertheless, the recent focuses on the potential role of PD as a risk factor for cardiovascular and cerebrovascular diseases bring new importance to this aspect of salivary analysis.[45]

10. Systemic diseases

Cardiovascular disease markers found in saliva, such as amylase is used for post-operative control of patients who had cardiovascular surgery. A study of Adam et al. showed that low levels of salivary amylase in the pre-operative stage of aorta aneurism is associated with an increase in mortality. Another study, done by Samaranayake in 2007 verified that alpha amylase salivary activity could be used as a good marker of catecholamines during the evaluation of patients in different stressful situations.[46]

Increased levels of salivary lysozyme are shown to be associated with hypertension, an early stage of cardiovascular disorders.[47] A co-relational study was done

to evaluate serum and salivary lipid profile in healthy individuals. There was a moderate level of correlation between serum and salivary TC, TGL, HDLC and VLDLC and there was a low and quite a small correlation between serum and salivary LDLC.[48]

Renal diseases, Walt *et al*[49] and Arregger *et al*[50] reported a series of salivary markers that were associated with end stage renal disease. The list of markers included Cortisol, nitrite, uric acid, sodium, chloride, pH, amylase and lactoferrin. In a subsequent study by these investigators, calorimetric test strips were used to monitor salivary nitrate and uric acid before and after hemodialysis.[51] It was suggested that a salivary test could be used by patients to decide when dialysis is required, thereby eliminating unnecessary visits to a dialysis clinic.[52] Salivary phosphate has been successfully used as a clinical biomarker for hyperphosphatemia, which is an important contributor to cardiovascular calcification in chronic renal failure.[52-54]

Psychological diseases, investigators have attempted to distinguish them using a variety of model systems that induce either stress or pain, and subjects are monitored for changes in salivary biomarkers. Typical markers that have been identified include salivary amylase, cortisol, substance P, lysozyme and secretory IgA

11. Genetic Disorders

Cystic Fibrosis (CF) is a genetically determined condition which is caused due to a mutation in the CFTR gene. Saliva is modified in CF patients. The CFTR protein is expressed in the epithelial cells of the parotid gland. The level of activity of salivary cathepsin-D in CF patients was significantly higher. The values of sodium, potassium, and chloride concentrations were significantly higher than healthy subjects. Salivary calcium, magnesium and lactate dehydrogenase levels were increased in CF patients compared with healthy controls.[47]

Ectodermal Dysplasia: The most common form of ectodermal dysplasia is the X-linked hypohidrotic ectodermal dysplasia (HED). Lexner et al. performed a study on WS flow and composition in males affected by HED and in female carriers. They found that there was reduced WS flow and concentration of inorganic constituents and total protein was high. However, the activity and the concentration of the alpha-amylase in the saliva were reduced.[47]

12. Occupational and Environmental Medicine

Salivary biomarkers play a role in the diagnosis of occupational stress (OS) and heavy metal toxin poisoning. OS is classified into two types. Chronic stress is associated with increased levels of salivary cortisol and decreased level of salivary IgA and lysozyme. Saliva chromogranin A and alpha-amylase are markers of acute stress.

Occupational toxins such as lead and cadmium are analyzed from the saliva. The concentration of cadmium in saliva is higher than in blood, but the level of salivary lead analysis is limited to higher levels of lead exposure poisoning.[47]

Contemporary advances in salivary diagnostics

Recent studies in SDs have demonstrated improvement of sensitivity and specificity using a combination of multiple biomarkers instead of a single biomarker in disease detection. Current efforts emphasize the discovery and validation of disease biomarkers and development of multiplexed nanotechnologies (lab-on-a-chip) for point-of-care and their ultimate translation into the real world through an industrial partner (Table 1).

Role of Salivary Biomarkers in diagnosis of Oral and Systemic diseases

Till date, most of the biomarkers have been identified from various body fluids. Among which blood and saliva are the most widely studied body fluids they contain reliable biomarkers for oral and systemic diseases. It is an informative body fluid containing an array of analyte (Protein, mRNA and DNA) that can be used as biomarkers for translation and clinical applications.[54]

The salivary biomarkers have been classified into Proteomic, genomic and microbiological biomarkers.[55] The wide continuum of molecules present in saliva provides valuable information for clinical diagnostic applications in clinical utility for followings:

1. Proteomic analysis
2. Genomic analysis
3. Transcriptome analysis

Table 1: Classification of Biomarkers

Proteomic Biomarkers			**Genomic Biomarkers**	**Microbial biomarkers**	**Other markers**
Immunoglubilins	Calprotectin	Kininase	Cathepsin C gene mutation	*Aggregatibacter Actinomycetemcomitans*	Calcium
Acid phosphatase	Caprylate esteraselipae	Lactoferrin	Collagen gene mutation	*Campylobactr rectus*	Cortisol
Alkaline phosphatas	Cathepsin B	Lactotransferrin	IL-1 polymorphis	*Mycoplasmas*	Hydrogen sulfide
Aspartate Aminotransferase	CD14	Lactate dehydrogenase	IL-10 polymorphisms	*Porphyromonas Gingivalis*	Methyl mercaptan
Aminopeptidases	Cystatins	Lysozyme	TNF Polymorphisms	*Prevotella intermedia*	Picolines
Beta-galactosidase	Elastase	MMP 1, MMP 2, MMP 3		*Peptostrepto coccus Micros*	PMNs
Beta-glucosidase	Epidermal growth Factor	MMP-8, MMP-9 MMP-13		*Prevotella nigrescens*	Pyridine
Beta-glucuronidase	Esterase	ICTP		*Treponema denticola*	
CRP	Fibronectin	Myeloperoxidase		*Tannerella forsythia*	
Alpha-glucosidase	Gelatinase	Osteocalcin		*Treponema socranskii*	
Histatin	Kallikrein	Osteonectin			
Mucins	Peroxidase	Osteopontin			

Salivary Proteomic Analysis

Human saliva is a plasma ultra filtrate and contains proteins either synthesized in situ in the salivary glands or derived from blood and contains biomarkers derived from serum, gingival crevicular fluid, and mucosal transudate. To date, researchers have identified 2,340 proteins in the salivary proteome, of which 20–30% are also found in blood[56], an encouraging indicator of the clinical utility of saliva as a diagnostic fluid. In contrast to the plasma proteome, in which 99% of the total protein content is contributed by 22 highly abundant proteins, the 20 most abundant proteins in WS constitute only 40% of the protein content.[57] This composition suggests that detecting biomolecules of clinical sensitivity and specificity in saliva should be practicable and easier than in blood. How molecules transport of blood in saliva may also be important for successful use of saliva as a diagnostic fluid. Lipophilic molecules such as steroid hormones passively diffuse into saliva, while water and electrolytes pass through the pores of acinar cells. Various peptides in blood move through protein channels, and large proteins are transported via pinocytosis.[58]

Proteomic Biomarkers

Development of analytical technologies in the post-genomic era has allowed for large scale identification of proteins/peptides (proteome) and ribonucleic acids (RNA; transcriptome), and their functions/structures in cells and

fluids. The high throughput proteomic studies have catalogued at least 1166 proteins in the major salivary gland secretions, of which 914 are recovered from parotid and 917 from submandibular/sublingual ductal saliva, with 57% of these proteins present in both glandular saliva.[59] The proteome of human minor salivary gland secretion showed 56 proteins.[60] More surprisingly, the salivary transcriptome (RNAs) has been discovered using microarray profiling in recent years and approximately 3000 messenger RNAs (mRNAs) are identified in cell-free WS. Most recently, the presence of microRNA (miRNA; ~50) was discovered in WS. Unlike mRNA, miRNA consists of 18–24 nucleotides transcribed from non-protein coding genes and regulates protein translation through an RNA-induced silencing complex (RIST).[61]

These advances have provided a large number of salivary molecular targets, e.g, proteins and RNAs, for disease biomarker discovery. Several investigators have already attempted to use high throughput technologies and current salivary proteomic and transcriptomic knowledge for biomarker discovery in the areas of oral and breast cancer[62], periodontal diseases[63, 64] cardiovascular disease and Sjögren's syndrome.[65, 66]

Traditional biochemical techniques such as LC, gel electrophoresis, capillary electrophoresis, nuclear magnetic resonance, MS, immunoassay, and lectin probe analysis have been widely used in saliva proteome work for identifying the proteins present in glandular saliva.[67, 68]

In the past few years, multiplex biomarker detection systems have emerged through remarkable progress in the development of lab-on-a-chip (LOC) and point-of-care (POC) technologies.[69] The goal of these efforts is to build automated, miniaturized, and multiplexed platforms for rapid assays and readout. In general, the principles of conventional ELISA and/or nucleic acid hybridization is applied often with either electrochemical sensors or a microbead reactor.[70, 71]

The UCLA School of Dentistry "UCLA Collaborative Oral Fluid Diagnostic Research Center" is the leading institute for this nano/micro-electrical-mechanical development. Alternatively, the microbead reactor system developed by the Texas-Kentucky Saliva Diagnostic Consortium consists of porous bead sensors consisting of a nano-net of agarose fibers serving as a chemical reaction matrix sequestering and concentrating analytes.[69]

The Texas/Kentucky Saliva Diagnostics Consortium is at the forefront of developing 3-D bead saliva/oral fluid diagnostics for cardiovascular, cancer, and periodontal diseases.[64, 70, 71]

Oral Fluid-based Lab-on-a-chip testing for detection of Acute myocardial infarction (AMI)

Coronary artery disease (CAD), a major component of cardiovascular diseases, causes 1 of every 5 deaths in the US in 2004. The survival by AMI is dependent on early diagnosis and emergency intervention and it is key for good prognosis.[3]

Currently, electrocardiogram (ECG) is standard equipment in emergency medical services (EMS) and is used as a diagnostic standard for emergency triage of patients with chest pain and/or unconsciousness. A typical ECG abnormality for an AMI is an ST segment elevation. Unfortunately, ECG alone only identifies ~35 % of all AMI cases admitted to the emergency department (ED) and misses the remaining 65%, that do not exhibit the characteristic ECG changes. The triage of potential AMI cases in the ED depends on supplemental blood testing that often includes cardiac troponins T and I (cTnT, cTnI), creatine kinases-MB (CK-MB), total CK and myoglobin (MYO).[70] However, these tests are, invasive and limited to the clinical laboratory setting and the few that have been developed for POC testing lack the analytical and clinical sensitivity and specificity to efficiently diagnose AMI.[73] So there is need to have a non-invasive test with the required analytical and clinical performance that could be used in an ambulance setting to minimize the time from diagnosis to treatment of AMI patients. Saliva presents itself as an ideal fluid in this situation.

A study has been done to evaluate the potential use of AMI biomarkers in saliva by collecting unstimulated whole saliva within 48 hours from more than 80 patients with a definitive diagnosis of AMI and compared with 80 healthy controls and assayed for 21 cardiac related proteins using conventional methodologies, such as LUMINEX, ELISA and Beckman Access instrumentation. Data gathered to demonstrate cardiac biomarkers/proteins such as C-reactive protein (CRP), myeloperoxidase (MPO), interleukins, matrix metallo-perteinase-9 (MMP-9), and cellular adhesion molecule-1 (sICAM-1), can be detected in saliva samples but, most importantly, demonstrated a capacity to differentiate healthy controls from AMI patients. Strikingly, it was showed, that AMI diagnosis was greatly improved with a combination of the ECG and AMI proteins in saliva.[70]

In parallel to discovering salivary AMI biomarkers, the critical steps for salivary marker measurement using NBC based technology sensor system developed and standardized a portable, modular device which includes ambulance sample collection, temperature, humidity, reagent stability, compromised light source.[69]

Exfoliative cytology based on nano-bio-chip sensor platform for oral cancer detection

Early diagnosis and intervention is the key for a better prognosis of cancer, underscoring the value of advanced screening and diagnostic techniques for oral cancer and, more importantly, pre-cancerous lesions.

Addressing this clinical need, research groups at Rice and the University of Texas Health Science Centers in San Antonio and Houston have adapted the bead-based NBC sensor system to establish a platform for whole cell analysis of tumor biomarkers in oral exfoliative cytological specimens. This technique and its potential use in oral diagnostics were recently described in a pilot study examining both molecular and morphological biomarkers associated with oral dysplasia and malignancy.[74]

Salivary proteomics for dental caries (DC)

Salivary proteins play a significant role in maintaining the oral health and prevent caries as stated by Mazengo *et al*.[75] A significant amount of salivary phosphopeptides (PRP1/3, histatin-1 & statherin) were associated with the absence of DC, emphasizing the importance of these peptides in the maintenance of tooth integrity.[76, 77]

In a recent study on early childhood caries, it was found that, a higher number of proline-rich protein bands significantly correlated among caries free subjects, substantiating the protective role of this protein, also a higher number of glycoprotein bands were observed in the WS of subjects with early childhood caries.[55]

Salivary proteomics for existing periodontal disease (PD)

Interlekin-1β (IL-1β) is a proinflammatory cytokine that stimulates the induction of adhesion molecules and other mediators which in turn facilitate and amplify the inflammatory response. Its levels correlated significantly with periodontal parameters after adjusting for the confounders. Moreover, combined levels of IL-1β and matrix metalloproteinase (MMP)-8 increased the risk of experiencing PD by 45 folds.[55]

MMPs, MMP-8, a key enzyme in extracellular collagen matrix degradation, derived predominantly from PMNs during acute stages of PD. Its presence significantly increased the risk of PD (odds ratios in the 11.3-15.4 range). MMP-1 (interstitial collagenase) also appeared to be activated in periondontitis[77] Additionally, higher levels of other MMPs, including MMP-2, MMP-3 and MMP-9, were also reported in the saliva of periodontitis patients.[55]

Immunoglobulin (Ig), In periondontitis patients they're shown to have higher salivary concentrations of IgA, IgG and IgM specific to periodontal pathogens compared with healthy patients. There was a great reduction in these immunoglobulins after treatment.[55]

Acid Phosphatase (ACP) and Alkaline phosphatase (ALP), A study found a significant positive correlation between salivary ACP and calculus formation. The increase in salivary ALP activity in periodontitis could be associated with alveolar bone loss, a key feature of PD. Esterase, Lysozyme, Lactoferrins, A statistically significant, positive correlation between salivary esterase and calculus formation was observed, the esterase activity of WS was higher in individuals with PD than in periodontally healthy subjects and reduction in levels following the treatment.[55]

Patients with low salivary levels of lysozyme are more susceptible to plaque accumulation, which is a risk factor for PD. Lactoferrin is strongly up-regulated in mucosal secretions during gingival inflammation and is detected at a high concentration in patients with PD compared with healthy patients. Numerous other proteomic markers, like Histatins, cystatins, Kallikreins & Kininogens, aminopeptidases, Aspartate transaminase, Glucosidase, Galactosidase and Glucuronidase and various bones remodeling proteins (Osteopontin, Osteonectin, Osteocalcin) are well known in periodontal diagnosis.[55]

Salivary proteases as biomarkers for premalignant and malignant oral lesions

Unfortunately, clinicians now lack tests which easily and reliably distinguish pre-malignant oral lesions from those already transitioned to malignancy. Bioinformatic analysis of exfoliated epithelial cells from subjects' saliva revealed increased myosin and actin abundance in malignant lesions as confirmed by western blotting. These findings provided a promising starting point for the development of non-invasive and inexpensive salivary tests to reliably detect oral cancer at an early stage.[78]

Oral lichen planus (OLP) is a chronic inflammatory mucosal disease with a cell-mediated immunological pathogenesis. Saliva from patients with OLP comprised various proteins. A total of 31 protein spots representing 14 proteins with at least twofold differences in abundance between OLP and controls were identified. In an another study it was found that the levels of salivary CD44s and CD44v5 (isoforms of CD44) from OLP patients were significantly higher than those from controls.[55]

IFN-γ and IL-4 levels in whole unstimulated saliva screened by ELISA in OLP patient showed a low-level IFN-γ but high-level IL- 4 expression profile, with a lower ratio of IFN-γ/IL-4 compared to healthy controls. Thus the salivary IL-4 level may be a fine biomarker reflecting the severity of OLP.[55]

A recent study stated that subtractive proteomics revealed several salivary proteins at differential levels between the OSCC patients. Five candidate biomarkers M2BP, MRP14, CD59, profilin and catalase were successfully validated using immunoassays on an independent set of OSCC patients and matched healthy subjects.

IL- 6 and IL-8 are involved in the pathogenesis of OSCC, and have been linked to increased tumor growth and metastasis, hence its levels could serve as informative biomarkers for OSCC in saliva.[79] A new study published by researchers at the UCLA School of Dentistry substantiates the effectiveness of measuring the microRNAs present in saliva to detect OSCCs. MicroRNAs are the molecules produced by cells that simultaneously assess the behavior of multiple genes and control their activity.

The role of proteomics in salivary gland neoplasm has been studied by Nakashima et al. Investigated the adenoid cystic carcinoma and detected 4 up-regulated and 5 down-regulated proteins[80], one study showed that there is an important relationship between some proteins, such as transketolase, dim1p, v-ha-ras oncogene, type I collagen pro alpha, tumor necrosis factor (ligand) superfamily member 4, Pirin and tumor metastasis.[81] The same Authors also investigated the differential expression of proteins in adenoid cystic carcinoma with lung metastasis and found that transketolase, modulator recognition factor 2, dim1p homolog, splicing factor (arginine/serine- rich 9) and v-ha-ras l oncogene were all hypoexpressed in poorly metastatic tumors and significantly upregulated in highly metastatic tumors.

Salivary proteomics for Sjögren's syndrome (SS)

SS is a systemic autoimmune disease, where immune cells attack and destroy the salivary and lacrimal glands. Mass spectrometry analysis showed 16 down-regulated and 25 up-regulated proteins in primary SS patients compared with matched healthy controls. These proteins reflected the damage of glandular cells and inflammation of the oral cavity in patients with primary SS.[55]

Systemic sclerosis, recently, Giusti et al. evaluated saliva protein profiles in these patients by proteomic approach and found that the level of all representative salivary proteins, except keratin, remained unchanged, only qualitative differences were observed between controls and patients. It was also detected previously and newly identified proteins in saliva of patients: some of these, such as keratin 6l, psoriasin, TPi, and arp2/3 complex, might have a pathological role in systemic sclerosis.[82]

Other clinical applications of proteomics: In anorexia, bulimia and celiac disease. Recently a study conducted on patients with autism for salivary peptides, for identifying differences between patients and age-matched controls. In particular, the phosphorylation level of four specific salivary phosphopeptides (statherin, histatin, acid proline rich proteins) was significantly lower in a subgroup of autistic patients.[83, 84]

Salivary Transcriptome analysis:

The Salivary Transcriptome (ST) offers an additional valuable resource for disease diagnostics. The first report of the ST demonstrated that the normal ST consists of about 3,000 mRNAs. Of particular importance is that of the 3,000 mRNAs, 180 are common between healthy subjects, constituting the normal salivary transcriptome core (NSTC).[85]

To demonstrate the diagnostic and translational potential of the ST, the UCLA group profiled and analyzed saliva from patients with oral cancer. Four genes from the NSTC (IL-8, ornithine decarboxylase, spermidine acetyltransferase and IL-1) were able to discriminate and predict, whether the saliva sample was from a patient with cancer or from a healthy subjects, with a sensitivity and specificity of 91%. The behavior of these ST biomarkers is consistent and their levels are significantly higher in saliva of patients with oral cancer compared to control subjects.

***Serum versus salivary Transcriptome*:**

Because saliva is not a mainstream diagnostic fluid, researchers at UCLA compared the clinical accuracy of

saliva with that of blood RNA biomarkers for oral cancer detection. They found four informative RNA biomarkers that have a sensitivity and specificity of 91 and 71 percent, respectively (ROC = 0.88). As explained above, the four salivary oral cancer biomarkers had a collective ROC value of 0.9518; this demonstrates clearly that for oral cancer detection, salivary transcriptome diagnostics have a slight edge over serum.[86] This example also points out an important fact with regard to the discovery and validation of biomarkers for disease diagnostics: the power of salivary biomarkers to discriminate and detect disease likely will be based on a panel rather than on a single biomarker.

Advantages of Salivary Transcriptome markers

While the human salivary proteome is still about two years away from being compiled, the salivary transcriptome of healthy subjects has been completed [85]. As a biomarker, RNA is as robust and as informative as any other analyte.Thus, salivary transcriptome offers the combined advantages of high throughput marker discovery via a noninvasive biofluidic method and high patient compliance.

Development of Technologies for Saliva-Based Diagnostics

In 2002, NIDCR initiated a research effort in the area of salivary diagnostics, and progress is being made toward developing technology viable systems that are suitable for commercialization. NIDCR funded seven awards for the development of microfluidics and microelectromechanical systems (MEMS) for salivary diagnostics (Figure 1).

NIDCR*-funded salivary diagnostic technology development and salivary proteome research groups.

SALIVARY DIAGNOSTIC TECHNOLOGY DEVELOPMENT	SALIVARY PROTEOME
University of Texas at Austin Eric Anslyn, PhD "www.cm.utexas.edu/directory/eric_anslyn/"	**University of California, San Francisco** Susan Fisher, PhD "www.salivarium.ucsf.edu/"
New York University, New York City Daniel Malamud, PhD, MA "www.nyu.edu/dental/research/faculty/malamud.html"	**The Scripps Research Institute, La Jolla, Calif.** John Yates, PhD "fields.scripps.edu/public/project/saliva/"
Sandia National Laboratories, Livermore, Calif. Anup Singh, PhD "www.ca.sandia.gov/chembio/microfluidics/staff/singh.html"	**University of California, Los Angeles** David Wong, DMD, DMSc "www.hspp.ucla.edu"
University of Washington, Seattle David Stahl, PhD "www.stahl.ce.washington.edu/index.html" Paul Yager, PhD "http://faculty.washington.edu/yagerp/"	
Tufts University, Medford, Mass. David Walt, PhD "chem.tufts.edu/faculty/walt/"	
University of California, Los Angeles David Wong, DMD, DMSc "www.saliva.bme.ucla.edu/"	

* NIDCR: National Institute of Dental and Craniofacial Research.

Figure 1: Development of microfluidics and microelectromechanical systems (MEMS) for salivary diagnostics

MEMS are integrated systems composed of mechanical elements, sensors, actuators and electronics on a common silicon substrate developed through microfabrication technology. These systems use small sample and reagent volumes coupled with integrated detection methods to perform an analysis. The seven NIDCR-supported awards focused on the development of microfluidic and MEMS technologies for measuring proteins, DNA, gene transcripts (mRNA), electrolytes and small molecules in saliva.[3]

T David et, all groups at the University of California, Los Angeles (UCLA) has developed a POC microfluidics system that can permit concurrent detection of multiple salivary analysts including proteins and nucleic acids.

The UCLA technology permits salivary analyte detection at high levels of sensitivity and specificity, without the need for a PCR for nucleic acid detection or an ELISA for protein detection. Clinical applications include oral and breast cancer and metabolic diseases (Eg, type2 diabetes).

Paul Yager, PhD, University of Washington, is developing an integrated microfluidic system for rapid, inexpensive and simultaneous measurement of multiple analytes in saliva that uses a simple disposable polymeric laminate format. The goal of this program is to detect low levels of hormones, drugs, metabolites, specific pathogens and markers of oral cancer, as well as protein markers of systemic disease.[3]

Commercially Available Saliva Tests

Two U.S. companies were early pioneers of oral diagnostics: Epitope, Inc. and Saliva Diagnostic Systems, Inc. They both commercialized saliva collection devices in the early 1990s, and in 1996 the Food and Drug Administration (FDA) approved Epitope's Orasure HIV test, the first test that used oral fluid to test for an infectious disease. The OraQuick HIV test, which takes only 15 minutes to detect the HIV antibodies in saliva via mouth swab.

Several companies outside the U.S. have commercial tests to detect drugs-of-abuse in a spit sample, including Cozart Biosciences, Securetec, and Mavand. Some of these companies send their kits via regular mail to customers, allowing individuals to collect their own saliva either in a cup or with a swab and then send the sample to a laboratory for analysis. Other tests target DNA in saliva. Canada-based DNA Genotek was the first company to commercialize a broad range of saliva collection tools for genotyping based on PCR, microarrays, and sequencing. Oral DNA Labs, a subsidiary of Quest Diagnostics, also offers two salivary tests in the U.S. in its CLIA-approved testing facility. My PerioPath is a DNA test that determines the risk of periodontal infections by detecting bacterial pathogens in saliva. OraRisk HPV is a salivary test that determines an individual's risk of developing HPV-related oral cancers. It identifies various HPV genotypes, including HPV 8, 11, 16, and 18.[87]

OraQuick, ADVANCE/Rapid HIV-1/2, Orasure HIV1, Periogard, Micro-plate EIA, ZRT Saliva Test, SALIVASCREEN 5 Professional, Q.E.D. Saliva Alcohol Test are a few of the examples of such commercially marketed chair side kits.[12]

Conclusion

As our knowledge of the biomolecules present in saliva grows, the potential applications for oral and systemic disease diagnosis will expand. While the scientific link between salivary biomarkers and oral diseases is clear, more studies are needed to delineate the mechanisms by which saliva reflects other systemic diseases. Furthermore, before saliva can become widely recognized as a reliable diagnostic fluid, we need to fully understand a number of important variables. First, we need to define the normal biological variability of biomolecules in saliva, such as diurnal rhythms, inter- and intra-subject variation, and age and gender effects. From an analytical standpoint, methodological variations caused by saliva sampling, handling, and storage conditions will need to be defined and analyte reference ranges in saliva need to be carefully documented. Since the salivary proteome is sensitive to both extrinsic and intrinsic factors

Saliva-based diagnostics present incomparable opportunities for research and commercialization opportunities because of increased understanding of genomics, transcriptomics and proteomics. At this stage it seems to be an extremely important possible tool for regular screening of larger populations. However, the path leading to practical and effective regular use of saliva as a powerful diagnostic tool is expected to be promising, but quite long. Hence, to execute all requirements for being saliva as a regular diagnostic tool in the day to day practice, further technological advancement and identification of robust and discriminatory sets of salivary biomarkers is necessary.

Taking all these aspects into consideration, it can be concluded that in the coming future, there are rich possibilities that salivary diagnostics can not only be used as a powerful tool for saving life but also to preserve those, which already have been saved. It will be a very helpful tool for population-based screening programs, confirmatory diagnosis, risk stratification, prognosis determination, and therapy response monitoring. Screening an entire population for a certain type of disease will be made possible in the near future by employing saliva diagnostics

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