

Salivary Biomarkers in the Diagnosis of Oral Diseases

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ABSTRACT

Introduction: Development of non-invasive diagnostic techniques to overcome the limitations of conventional diagnostic techniques such as serum analysis has gained much momentum in the recent years. Advances in the “omics” disciplines have led to the discovery of a slew of molecules such as genetic variations, proteins, RNA transcripts, and small metabolites that might aid in the early detection of at-risk persons. Saliva, commonly called the “mirror of the body,” appears to be an appealing alternative for laboratory or clinical assessment, therapy monitoring, and prognosis evaluation in both oral and systemic disorders. However, due to the fairly recent nature of the field, sheer volume of studies being done to identify biomarkers and the absence of a structured database for reference, clinical translation of salivonomics research is limited.

Objective: This review aims to enumerate all the biomarkers and diagnostic kits available for the salivary diagnosis of microbial, autoimmune, inflammatory and malignant lesions of the oral cavity.

Materials and Methods: Review articles and original research papers published in various electronic databases like PubMed, Cross reference, Google scholar, and data collected from books are compiled in this review article.

Results and Conclusion: This review gives an overview of the role of salivonomics in the diagnosis of some of the common diseases affecting the oral cavity, thereby attempting to bridge the gap between research and clinical dental practice.

Keywords: Salivonomics, Point-of-care devices, Dental caries, Periodontitis, Oral malignancies

INTRODUCTION

Saliva is a hypotonic solution secreted by the salivary glands, and contains secretions from the gingival crevice and the oral mucosa. It is known as the “mirror of the body” as it reflects the health and wellbeing of the body as a whole.^{1,2} Saliva as a biofluid has been the focus of research for quite some time as it contains biomarkers that are generated in the oral cavity as well as, those found in the serum. This coupled with the non-invasive nature of saliva collection and relative ease of sample handling and processing has made saliva a very attractive solution for laboratory or clinical diagnosis, monitoring of treatment effects and assessment of prognosis in a myriad of diseases. The recent advancements in the field of molecular and nanotechnology coupled with the enormous amount of work being done in the field of salivary diagnostics has led to the development of a new branch in diagnostic medicine termed “Salivonomics”.³

Saliva performs a variety of roles due to its unique physicochemical and biological characteristics, as well as its intricate chemical composition. Along with breath, gingival crevicular fluid, and other factors, it is crucial for preserving the homeostasis of the oral cavity. The balance in the oral microenvironment can be upset by slight differences in saliva quality or quantity, which can result in a variety of illness symptoms. Therefore, by using the biomarkers found in saliva, these diseases may be identified, their responses to treatment analysed, and in certain situations, they can be prevented.

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This article attempts to shed light on the versatile application of saliva as a diagnostic tool in the early identification of various oral infections, inflammations, malignancies and also orofacial pain and autoimmune disorders that present with a more indistinct set of signs and symptoms.

ORAL DISEASES CAUSED BY BACTERIA

There are over 700 different kinds of microorganisms present in the oral cavity, of which 200 are bacteria. Less than 50% of the bacterial population in the oral cavity is non-cultivable, making diagnosis difficult. Salivary biomarker analysis is recently being researched extensively as a more effective

tive alternative to aid in the diagnosis and treatment of these diseases⁴. Dental caries and periodontal disease are the 2 major bacterial infections affecting the oral cavity. As the nature and constituents of saliva produced is stated as a major propagating factor of these disease, its analysis can prove beneficial in the early diagnosis and prevention of the same.

1. Dental caries

Dental caries is defined as a biofilm-mediated, diet modulated, multifactorial, non-communicable, dynamic disease resulting in net mineral loss of dental hard tissues.⁵ Sugary diets, improper brushing habits, bad oral hygiene, and a lack of awareness are a few of the things that have contributed to an increase in dental caries. Along with this, changes in the lifestyle and eating habits, socioeconomic standing, and socio-demographic factors also have a role in the development of caries.⁶ In general, diagnosis of caries is carried out through clinical examination and radiographic investigations⁴. Dental caries management has recently shifted in favour of a risk-based treatment approach that favours prevention-focused, minimally invasive procedures carried out as part of continuous, personalized patient care prediction.⁷ Saliva has been suggested as an ideal diagnostic tool for risk assessment, as it plays a major role in the physiology and pathology of the oral cavity. A shift in oral microbiota from healthy to cariogenic is indicated by high levels of *S. mutans* and *Lactobacillus*.⁸

Both quantitative and qualitative analysis of saliva have been used widely to determine the risk of dental caries and other oral diseases. In conditions of xerostomia, where there is

drastic decrease in unstimulated [$<0.1-0.2$ ml/min] and stimulated [$<0.4-0.7$ ml/min] saliva, a proportional increase in the caries incidence is seen⁹. This is attributed to the decrease in salivary buffer capacity which eventually leads to a decrease in pH below critical pH and result in the formation of dental caries¹⁰. Low salivary flow rate also increases the total salivary protein levels and decreases mucins, stimulating the formation of heterotypic complexes involving salivary components such as high-molecular-weight mucin glycoprotein-1 [MG-1], amylase, histatin 1, acidic proline-rich protein-1 [PRPs], and statherin, ultimately resulting in plaque formation and dental caries. The decrease in mucin content is also seen to increase caries susceptibility as it impedes the production of acquired enamel pellicle, making the tooth surface more prone to demineralization¹⁰⁻¹³. Recent studies have also shown a decrease in salivary immunoglobulin A, amylase, lactoferrin, lipocalins and cystatins which indirectly leads to an increase in caries incidence because of the lack of inhibition of cathepsin enzyme¹¹.

2. Periodontal Diseases

Periodontitis is defined as an inflammatory disease of supporting tissues of teeth caused by specific microorganisms or groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with periodontal pocket formation, gingival recession or both¹⁴. It is a public health concern due to the high frequency of the condition in adults, adolescents, and older people. Periodontal disorders have a number of risk factors associated with them, including smoking, bad oral hygiene, diabetes, medication, age,

Table 1: Biomarkers in periodontal diseases

| DISEASE | BIOMARKERS | PRESENTATION |
|--|---|---|
| DENTAL CARIES | Salivary flow | Decreased ⁹ |
| | Total protein levels | Increased ¹⁰ |
| | Mucin | Decreased ¹² |
| | Salivary immunoglobulin A, amylase, lactoferrin, lipocalins and cystatins | Decreased ¹¹ |
| PERIODONTAL DISEASE | | |
| Gingivitis and periodontitis | Salivary macrophage inflammatory protein-1 α , matrix metalloproteinase-8,9, interleukin [IL]-1 β , IL-6, prostaglandin E2 and tumour necrosis factor [TNF]- α 1. | Increased ²² |
| | Proteolytic granulocyte enzyme elastase, protease inhibitor alpha 1-antitrypsin, and elastase inhibitor alpha 2-macroglobulin. | Increased ²³ |
| Progression of gingivitis to periodontitis | Immunoglobulin Molecular chaperone hsp70, Cystatin S, Salivary amylase, Calprotectin, Histatins, Lysozyme, Lactoferrin, Defensins, Peroxidases, Proline-rich proteins, Mucins | Decreased ^{24,25} |
| Periodontitis | Cystatins, Lysozyme | Decreased ²⁶ |
| | Toll-like receptor-4, IL-18, uric acid, aspartate transaminase and Procalcitonin | Increased, Corelates with clinical measurements ²⁷ |
| Chronic periodontitis | Superoxide dismutase | Increased, Corelate with clinical presentation ²⁸ |
| Aggressive periodontitis | Adrenomedullin, Nitric oxide | Increased levels ²⁹ |



hereditary factors, and stress¹⁵. Periodontitis is also known as the sixth complication of diabetes mellitus¹⁶. Evidence suggests that periodontal changes are the first clinical manifestation of diabetes¹⁷. Hence, simple, accurate and non-invasive diagnosis of periodontitis could go a long way in the early detection and intervention of diabetes mellitus.

Saliva can act as both a vector and a reservoir for bacterial colonisation¹⁸. The presence of specific bacterial species in saliva can indicate the presence of those organisms in dental plaque and periodontal pockets¹⁹. Researchers are comparing salivary proteomic profiles in patients with periodontitis and healthy individuals, finding unique profiles with changes in salivary proteins in the presence of periodontal inflammation, which could aid in enhancing periodontal diagnosis²⁰. Several studies have been conducted to identify periodontal disease causing pathogens such as *P. gingivalis*, *T. denticola* and *T. forsythia* and their correlating biomarkers in the saliva²¹.

Table 2: Biomarkers in oral inflammatory based lesions

| DISEASE | BIOMARKER | PRESENTATION |
|-------------------------------|---|----------------------------|
| Recurrent Aphthous Stomatitis | Cortisol | Increased ³⁷ |
| | TNF- α , PEG-2, VEGF, IL-6 | Increased ³⁸ |
| Burning Mouth Syndrome | Salivary flow, viscosity | Decreased ⁴¹ |
| | Chloride, Potassium, Phosphorus | Increased ⁴² |
| | Cortisol, α -amylase, Low molecular weight protein | Increased ^{41,42} |

Table 3: Biomarkers in oral autoimmunity-based lesions

| DISEASE | BIOMARKERS | PRESENTATION |
|-----------------------|---|-------------------------|
| LICHEN PLANUS | | |
| Oral Lichen Planus | Cortisol, IgA, | Increased ⁴⁶ |
| | CD14, Toll-like receptor | Increased ⁴⁷ |
| | MMP-8, CTX-1 | Increased ⁴⁸ |
| | Defensin-1 | Increased ⁴⁹ |
| | Urinary prokallikrein | Increased ⁵⁰ |
| | Palate, lung, and nasal epithelium carcinoma associated protein | Decreased ⁵⁰ |
| | Complement C3c, fibrinogen fragment D | Increased ⁵³ |
| | Cystine SA | Decreased ⁵³ |
| Erosive Lichen Planus | Nitric oxide | Increased ⁵⁴ |
| Pemphigus Vulgaris | Anti- Desmoglein 1 and 3 | Increased ⁵² |

3. Oral Diseases Caused By Fungus

In healthy individuals, the oral cavity includes commensal microbiota that coexists in a delicate immunophysiological balance, of which the mycobiome forms an important part. Oropharyngeal candidiasis [OPC] is a common oral disease caused by culturable and non-culturable resident fungus, most commonly, *Candida albicans*⁴. Oral mycoses, including OPC, are traditionally diagnosed with an oral clinical examination and the collection of oral specimens [swab, sputum, or saliva] for specimen isolation and histopathological diagnosis of causative species^{30,31}. Histatin 5 and β -defensin, which have fungicidal characteristics, are found in human saliva and can be used to detect and treat oral candida infections^{32,33}. Commercial ELISA kits to detect *Candida*'s mannan antigen in oral rinse solutions are under experimentation³⁴. Salivary IgA or IgG antibodies to *Candida* have also been detected experimentally, although immunodiagnosis remains tricky due to discrepancies in sensitivity and specificity of different assays when detecting distinct *Candida* antigen preparations³⁵.

4. Oral Inflammatory Based Lesions

4.1 Recurrent Aphthous Stomatitis

Recurrent aphthous stomatitis [RAS] have an unclear aetiology, but histological evidence reveals non-infectious, nonspecific inflammation of mucosal tissue that begins in subepithelial connective tissue³⁶. Numerous studies have been conducted to analyse the salivary proteome for a biomarker diagnose RAS, salivary cortisol levels being the most common, which is significantly increased³⁷. Studies also have estimated elevated levels of proinflammatory cytokines TNF- α , PEG-2, VEGF, IL-6 in the saliva samples of those suffering from RAS as they trigger production of various inflammatory molecules that can further precipitate epithelial damage^{37,38}.

4.2 Burning Mouth Syndrome

Burning mouth syndrome [BMS] is defined as a burning sensation or other dysesthesias of the oral mucosa that is not accompanied by aberrant clinical or laboratory results.³⁹ Although the aetiology of the disease is still unknown, several local, systemic and psychological factors have been attributed to it⁴⁰.

The quantitative evaluation of salivary changes in the affected individuals has shown a significantly lower unstimulated salivary flow rates [xerostomia] and viscosity⁴¹. As postmenopausal women are most commonly affected by the disease, saliva was analysed for changes in the trace elements composition which was significantly increased⁴². Proteomic salivary analysis of glossodynia patients revealed an increase in the levels of cortisol, α -amylase, total protein concentration, particularly low molecular weight protein expression^{41,42}. More research is required in this field as most of the present literature is contradictory and inconclusive in nature.

5. Oral Autoimmunity Based Lesions

5.1 Oral Lichen Planus

Oral lichen planus [OLP] is a chronic, T-cell mediated, mucocutaneous lesion affecting the oral mucous membranes⁴³. As the lesions mimic other vesiculo-bullous diseases, histopathologic diagnosis is considered gold standard⁴⁴. The recent COVID-19 pandemic has brought about a rise in the prevalence of OLP. Patients with COVID-19 may experience an increase in



Table 4: Biomarkers in oral malignancies

| DIAGNOSTIC TOOLBOX | BIOMARKERS | PRESENTATION |
|-------------------------------------|--|---|
| ORAL SQUAMOUS CELL CARCINOMA | | |
| Genomics | C-myc, c-Fos, C-Jun | Increased ⁴ |
| | P16 | Decreased ⁴ |
| | TP53, NOTCH1, PIK3CA and CDKN2A genes | Point mutations seen ⁶⁰ |
| | Tumor DNA | Translocation mutation ⁶⁰ |
| | P53 antibodies | Expressed ⁶³ |
| Transcriptomics | Transgelin mRNA | Increased ⁶⁴ |
| | Integrin mRNA- ITG- α 3, ITG- α 5, ITG- β 1 | Increased. ⁶⁵ |
| | mRNA of IL-1 β , IL-8, HA3, DUSP1, OAZ1, S100P, and SAT | Three-fold increase ⁶⁶ |
| | miR-125a and miR-200a | Decreased ⁶⁷ |
| | OncomiR-27b | Increased ⁶⁸ |
| | TSmiR-136 | Decreased ⁶⁸ |
| | miR122-5p and miR-146a-5p | Increased expression and differentiates between TNM stage 2 and 3 ⁶⁹ |
| | miR124-3p | Decreased expression and differentiates between TNM stage 2,3, and 4 ⁶⁹ |
| | miR-92a-3 | Decreased expression seen in TNM stage 3 ⁶⁹ |
| | miR 21-5p | Increased ⁷⁰ |
| | let-7c, miR-99a, and miR 100-5p | Decreased ⁷⁰ |
| | miR302b-3p and miR512-3p | Expressed only in OSCC ⁷¹ |
| Proteomics | α -1- β -glycoprotein and complement factor β proteins | Expressed in OSCC ⁷² |
| | Cystatin S, Parotid secretory factor and Poly-4-hydrolase beta-subunit proteins | Absent in OSCC ⁷² |
| | Beta fibrin, S100 calcium binding protein, transferrin, immunoglobulin heavy chain constant region gamma and cofilin-1 | Increased ⁷³ |
| | IL-10, IL-13 | Elevated in poorly differentiated OSCC ⁷⁴ |
| | TNF- α | Elevated in moderately and poorly differentiated OSCC ⁷⁵ |
| | IL-1RA | Elevated in well differentiated OSCC ⁷⁴ |
| | MMP-1, KNG-1, ANXA-2 and HSPA-5 | Elevated and bifunctional panel for PMD risk assessment and the detection of OSCC ⁷⁶ |
| | PRDX-2, ANXA-1, and ZA2G | Elevated levels seen in early OSCC ⁷⁷ |
| Metabolomics | Priopionyl choline+ N-acetyl-L-phenylalanine + sphinganine + phytosphingosine, and S-carboxymethyl-L-cysteine | Panel is highly specific and sensitive for OSCC ⁷⁸ |
| | Ornithine + o-hydroxybenzoate, and ribose-5-phosphate | Elevated, Early diagnostic panel for OSCC ⁷⁹ |
| SALIVARY GLAND TUMORS | | |
| Proteomics | Maspin and stathmin | Increase in levels correlated with histological grade of tumor ⁶¹ |
| | Transketolase, modulator recognition factor 2, Dim1p homolog, splicing factor and the v-Ha-ras I oncogene | Increased in highly metastatic tumors ⁶² |
| | Type I collagen pro- α and tumour necrosis factor | Decreased in metastatic tumors ⁶² |
| | Retinal home box protein | Expressed in metastatic tumors ⁶² |
| | Pirin | Expressed in non-metastatic tumors ⁶² |



OLP or an aggravation of pre-existing lesion due to altered T-cell responses, cross-reactive antibodies, elevated cytokine levels, altered immunological permeability barrier, and Vitamin D deficiency⁴⁵. The stress caused by the global pandemic is also a possible causative factor of the disease.

Salivary analysis is now being considered as a non-invasive alternative for the diagnosis of OLP. Considering the aetiology inflammatory molecules such as C-reactive protein, IgA, and matrix metalloproteinase 8 demonstrate upregulation in the saliva of OLP patients⁴⁶⁻⁴⁸. Few studies have shown an increase in defensin-1, and urinary prokallikrein levels and a decrease in palate, lung and nasal epithelium carcinoma associated protein [PLUNC]^{49,50}.

5.2 Pemphigus Vulgaris

It is defined as “An autoimmune intraepithelial blistering illness that affects the skin and mucous membranes”. It is caused by circulating autoantibodies that target the keratinocyte cell surface. Microscopic identification of suprabasal clefting, acantholytic cells and deposition of IgG antibodies and complements in skin and mucosal biopsies through direct immunofluorescence is the preferred method of diagnosis⁵¹. In the light of the development of salivomics anti- Dsg ELISA kits may now be used to detect anti- Dsg 1 and anti- Dsg 3 antibodies for diagnosis, assessment of severity, and monitoring disease activity in pemphigus⁵².

6. Oro- Facial Pain

Orofacial pain is a sensory sensation that occurs inside a specific anatomical location and is linked to a variety of chronic orofacial conditions, including: Atypical odontalgia, burning mouth syndrome, persistent idiopathic facial pain, neuralgia of the head and neck, TMJ myalgia and arthralgia⁵⁵. As all the above-mentioned conditions present with pain as their major symptom, it is hard to differentiate and treat.

Saliva can be utilised to diagnose these conditions as studies have found an increase in the amount of substance P, cortisol and biomarkers of oxidative stress. Levels of glutamate, an important pain mediator in the peripheral tissues, were also found to be elevated^{55,56}.

7. Oral Malignancies

7.1 Oral Squamous Cell Carcinoma

The most common cancer of the oral cavity is oral squamous cell carcinoma [OSCC], which accounts for more than 90% of verified cases and is among the top 10 cancers worldwide⁵⁷. Epidemiological data have shown a rise in the incidence and mortality of oral cavity cancers in a number of nations⁵⁸. The latter is correlated with tobacco use, alcohol use, oral pathogen infections, environmental variables, and poor oral hygiene⁵⁹. A special tool for diagnosing OSCC and all of its stages of development, including the early stage, invasiveness, recurrence, and therapy, is the discovery of biomarkers in saliva. It is doubtful that a single biomarker will always detect OSCC due to the diversity of carcinogenic pathways, tumour heterogeneity, and significant risk factor variance. Combinations of biomarkers are therefore more likely to improve diagnostic validity⁶⁰. These biomarkers were identified using molecular, transcriptomic, genomic, proteomic, metabolomic, and phenotypic techniques. Before being employed in clinical diagnostics to assist with early cancer detection, risk assessment, and treatment response, these biomarkers must be further developed and confirmed⁴.

7.2 Salivary Gland Tumors

Proteomics has been utilised to identify malignancies of the salivary glands and track the spread of tumours. Upregulation of 4 salivary proteins- stathmin, maspin, fibrin beta, sialic acid binding immunoglobulin-like lectin 8, along with downregulation of 5 proteins- enoyl coenzyme A hydratase short chain 1, serin proteinase inhibitor B 1, superoxide dismutase 2, aminolevulinic acid delta-dehydratase, pro-apolipoprotein was found after analysis of the salivary protein composition in patients with salivary gland neoplasms. Among these, the increase in maspin and stathmin levels could be correlated with the histological grade of the tumour⁶¹. One of the main uses of salivary proteomics has been the quest for salivary biomarkers that can effectively detect distant metastases. Highly metastatic tumours were shown to have much higher levels of transketolase, modulator recognition factor 2, Dim1p homolog, splicing factor [arginine/serine rich 9], and the v-Ha-ras oncogene⁶².

Table 5: Point-of-care devices for the salivary diagnosis of oral diseases.

| DISEASE | BIOMARKER | POC DEVELOPED |
|-------------------|---|--|
| Periodontitis | Porphyromonasgingivalis | P. gingivalis saliva kit ⁸³ |
| | Proteins [Dipeptidyl peptidase etc.], metabolites and DNA | Integrated Microfluidic Platform for Oral Diagnostics [IMPOD], lab-on-a-chip [LOC] ⁸⁴ |
| Sjogrens Syndrome | Salivary anti-Ro60 and anti-Ro52 Antibody profiles | Luciferase Immunoprecipitation Systems [LIPS] ⁸⁵ |
| Oral Cancer | microRNA-200a | Electrical controlled magnetic EC Sensor ⁸⁶ |
| | IL-8, IL-8mRNA | Electrochemical magneto biosensors ⁸⁷ |
| | Oral cancer overexpressed 1 [ORAOV1] | Electrochemical sensor using endonuclease target recycling amplification ⁸⁸ |
| | Epidermal growth factor receptor [EGFR] | Electric field induced release and measurement method ⁸⁹ |
| | Uric acid | Wireless mouthguard enzymatic biosensor ⁹⁰ |



8. Recent advances

The rapid advancements in the field of biomedical engineering have further revolutionised the field of salivary diagnostics with the advent of accurate and easy-to-use portable platforms capable of detecting the presence and absence of disease, outside the laboratory at the patient's bedside. These are point of care devices. A minimum risk of infection with no mental or physical suffering is of essential importance to take into account when developing point of care (PoCT) devices, in addition to automation, integration, multiplexed detecting ability, quick analysis, small sample size, and little training as major goals of modern medicine⁸⁰. Salivary diagnostics are being combined with microfluidics or micro/nanoelectromechanical systems [MEMS/NEMS] in a number of innovative technologies⁸¹. The simultaneous detection of many biomarkers is made possible by the recently created PoCT tests for "lab-on-a-chip," which facilitates the diagnosis of a number of human diseases⁸².

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