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REVIEW ARTICLE **OPEN ACCESS**

Salivary Biomarkers for Periodontal Diseases and Personalized Monitoring-A Critical Review

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ABSTRACT
Early diagnosis of disease plays a crucial role to reduce the severity and possible complications of the disease process. To overcome this challenge, salivary biomarkers are rapidly gaining popularity as a diagnostic tool. In the field of periodontology, a conscious effort is made to propose periodontal-therapeutic tools so as to monitor the disease at the earliest stage without allowing it to progress and cause systemic adverse effects. This review highlights the various salivary biomarkers used for personalized monitoring of periodontal diseases.
Keywords: *periodontitis, biomarkers.*

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INTRODUCTION

Periodontitis is an inflammatory disorder linked by Gram-negative anaerobic bacterial infection and characterized by composite immune reaction as a response to bacterial load. This disease is based on the biofilm which develops on the surface of the tooth, and a sudden shift in the composition of the oral microbiome that paves the way for opportunistic pathogens to induce disease outbreaks and progression [1].

The transition from gingivitis to periodontitis initiates when the periodontal pathogens such as *Porphyromonas gingivalis* (*P. ginigvalis*), *Aggregatibacter actinomycetemcomitans* (A. a), *Tannerella forsythia (T. forsythia), Treponema denticola (T. denticola),* and spirochetes, increase their population in the subgingival biofilm [2]. Alterations in the bacterial composition of the biofilm are responsible for disturbing the normal symbiotic relationship between the host and its resident microbiota leading to an altered host immune response. [3], This response is a "double-edged sword," which is responsible for aberrant immune response and also for periodontal tissue breakdown proportional to disease severity. The persistence of biofilm and aberrant immune response influences the patient's susceptibility to developing periodontitis.

PERIODONTITIS AND SYSTEMIC COMPLICATIONS

The pathogenesis of periodontitis involves a complex cascade initiated by the bacteria when it binds with the host cells' receptors. Pathogen mediates a series of events leading to the recruitment of inflammatory cells at the infection site to release various pro-inflammatory mediators and enzymes such as C-reactive protein (CRP), interleukin (IL)-1b, IL-6, tumor necrosis factor (TNF)-a, and matrix metalloproteinases (MMP). (4). This triggers the phagocytosis of bacterial cells, followed by the apoptosis of phagocytic cells and the resolution of inflammation. Even after the removal of the pathogen, the inflammation cascade fails to switch off and leading to uncontrolled chronic inflammation. Consequently, the rate of periodontal tissue destruction is accelerated and the cumulative increase in inflammatory cytokines, acts as a possible risk factor for several systemic diseases.

Thus, the susceptibility to periodontitis appears to be determined by the host response; specifically, the magnitude of the inflammatory response and the differential activation of immune pathways (Figure 1).

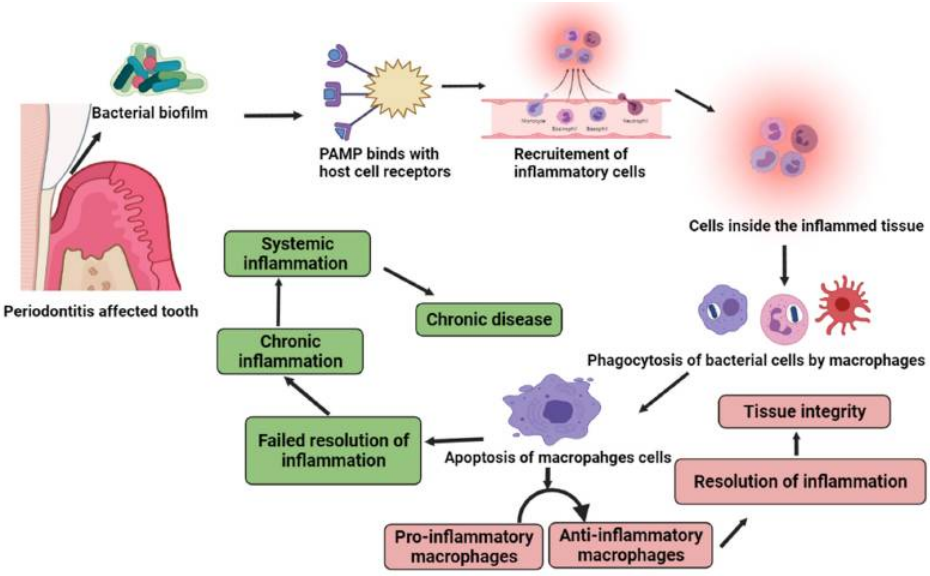

Figure 1 – Pathogenesis of Periodontitis

PERIODONTITIS AND SYSTEMIC DISEASES

Periodontitis and systemic diseases are complex diseases, linked by an established bidirectional relationship. Recently there has been much emphasis on the 'two-way' relationship between systemic disease and periodontitis [5]. That is, not only are conditions like diabetes, coronary artery disease, end-stage renal disease, arthritis, etc risk factors for periodontitis, but periodontitis could have a negative effect on metabolic control. The evidence supports the notion that improvements in metabolic control can be anticipated following effective treatment of periodontitis. The mechanisms by which this occurs are not yet clear, but probably relate to reduced systemic inflammation following the treatment and resolution of periodontal inflammation.

Many studies have suggested the possible mechanism was the focal infection in the oral cavity provoking systemic complications. The idea of a possible link between periodontitis and systemic diseases is that periodontal bacteria enter the systemic circulation through any dental procedure leading to bacteremia. [6]. The bacterial metabolic products such as endotoxins and gingipains released, trigger the systemic inflammatory responses by damaging distant organs like vascular endothelium, joints, respiratory tracts, etc.

Association between diabetes and Periodontitis

The elevated inflammatory state in diabetes contributes to both microvascular and macrovascular complications, causing activation of pathways that increase inflammation, oxidative stress, and apoptosis. A dysregulated immune system associated with the production of advanced glycation end-products (AGEs), insulin resistance, and hyperlipidemia [7] weaken the immune responses leading to the production of inflammatory mediators and reactive oxygen species that promote oxidative stress and endothelial cell damage, which is a complication of diabetes. AGE production causes burst in PMN release which leads to localized tissue damage in periodontitis. In addition, the gram-negative periodontal pathogens present in the ulcerated pocket epithelium also serve as a local source for the production of inflammatory mediators. All these factors contribute to the up-regulation of periodontal inflammation in diabetic patients.

Association between coronary artery disease and periodontitis

Chronic vascular disease (CVD) starts with the accumulation of low-density lipoprotein (LDL) that changes cellular permeability which provokes a state of secondary inflammation leading to endothelial dysfunction. Periodontal pathogens stimulate a systemic inflammatory response causing enhanced secretion of intravascular plasma proteins such as CRP protein that induces atherogenic mechanism by their action on endothelial cells, altered lipid metabolism, and increasing oxidative stress [10]. These cytokines contribute to destructive events in CVD and periodontal tissue.

Association between end-stage renal disease and periodontitis

Chronic kidney disease (CKD) is a state of reduced glomerular filtration rate, increased urinary albumin excretion, or both, that requires renal replacement therapy, including peritoneal dialysis, hemodialysis, or kidney transplantation [8]. Many studies have suggested the association between periodontitis and CKD that periodontal pathogens and inflammatory cytokines from the infected periodontium travel via the bloodstream and affect the endothelial function of nephrons. Due to general debilitation and depression

of the immunological system, CKD patients are more susceptible to periodontal disease. The altered saliva composition and persistent low-grade inflammation mechanistically influence the onset and/or progression of periodontal disease [9].

Association between periodontitis and cancer

Cancer-associated inflammation is similar to chronic inflammation by stimulating an antitumor immune response. The possible mechanism behind periodontitis-induced cancer is a failure in periodontal inflammation resolving mechanism, inflammation-induced production of reactive oxygen species, reduced immune surveillance, or genetic alteration.

A possible link established between periodontal disease and systemic disorders includes inflammatory pathways such as increased levels of white blood cells (WBC), CRP, fibrinogen, and proinflammatory cytokines. Evidence from most of the observational studies suggests that the examination of the levels of inflammatory chemokines such as IL-1,8, 10, MMPs can be used to assess periodontal health status.

PROMISING SALIVARY BIOMARKERS FOR EARLY DIAGNOSIS OF PERIODONTITIS AND THEIR COMBINATION

Pathologically, periodontal disease is characterized by persistent inflammation, connective tissue breakdown, and alveolar bone destruction. Usually, diagnosis is made on parameters, such as plaque index, bleeding on probing, periodontal pocket depth, attachment level, and radiographic assessment of alveolar bone loss. Bleeding on probing is still considered the best predictor of disease activity, however, it is not specific enough and can reveal too many false positives also. The identification of susceptible individuals or sites at risk from disease, and the diagnosis of active phases of periodontal disease, represent a challenge for both clinicians and oral health researchers. (Table 1)

Due to a lack of knowledge on understanding the exact phenomena that trigger the cascade of events leading to tissue destruction, this gap in knowledge can be bridged by performing studies to experiment whether all markers are potentially associated with initiating the disease process by salivary genomics, proteomics, and other state-of-the-art diagnostic techniques. Basically, periodontitis develops with elevated levels of several inflammatory mediators, such as C-reactive protein, interleukin-6, etc. The predictive value of salivary biomarkers in the onset and progression of periodontitis needs to be further exploited. The National Institute of Dental and Craniofacial Research encourages the development of non-invasive screening tests using saliva for clinical point-of-care that enables rapid quantification of oral disease.

Moreover, currently, the advanced research in periodontology is to evaluate the plausible risk of disease severity under each systemic condition by determining biomarkers for screening and predicting the early onset of disease or evaluating the disease activity and the efficacy of therapy. A self-administered home test that serves as a screening tool for periodontal diseases could play an important role in making individuals aware of the pathological process that occurs within the oral cavity. To overcome the hidden lethal threat before the disease becomes complicated, salivary biomarkers specific for the unique physiologic aspects of periodontal diseases can be used to monitor.

The biochemical analysis of saliva is used especially for estimation of the risk of disease onset and severity, monitoring of disease progression, and evaluation of therapeutic efficacy for premalignant and malignant oropharyngeal lesions as well as infectious diseases of the oral cavity. Common salivary biomarkers shown to be correlated with disease onset and activity falls under three key features of the pathogenic processes in periodontal disease – inflammation, collagen degradation, and bone turnover are pro-inflammatory cytokines, immunoglobulins, enzymes constituents of gingival crevicular fluid, and bacterial components or products [11]. (Table 2)

The correlation between salivary biomarkers and clinical features is basically based on three aspects of periodontitis – inflammation, collagen degradation, and bone turnover [12]. (Table 3)

Diagnostic precision with the combination salivary biomarkers for periodontitis vs. healthy gingiva, high sensitivity of 94%, and a specificity of 100%.

Table 1 – Diagnostic tool to measure periodontal disease status

Diagnostic level	Pathology	Diagnostic tool
Clinical	Attachment loss	Periodontal probing
	Bone loss	Radiographs
Tissue	Downgrowth of junctional epithelium, bone and connective tissue loss	Immunohistochemistry
Cellular	Inflammatory cell (PMNs, macrophages) activation, osteoclast activation	ELISA, immunohistochemistry
Molecular	Activation of receptors for endotoxin, Toll-like receptors	PCR: DNA-DNA hybridization, laser capture microdissection

Table 2 – salivary biomarkers for periodontal disease

Pro-inflammatory cytokines	Immunoglobulins	Enzymes	Bacteria derived enzymes	Others
IL-1β,	IgM	Aspartate aminotransferase	Elastase	Myeloperoxidase
IL-8,10	sIgA	MMP – 8,9,13	Trypsin like proteases	Osteocalcin
PGE-2	IgG	Lactate dehydrogenase	Aminopeptidases	Osteonectin
TNF-α		Lysozyme	Dipeptidyl peptidases	Osteopontin
β glucuronidase		Acid phosphatase		Cathepsin B
		Alkaline phosphatase		Pyridine, Picolines

Table 3 – Promising salivary biomarkers of periodontal disease for personalized monitoring

Biomarker	Function	Reference	Observation
MMP-1 MMP-8 and MMP-13	Remodeling and degradation of extracellular matrix components	15, 17	degradation of gingival and periodontal ligament collagen and bone destruction
TNFα, PGE2	Initiate inflammatory response, Osteoblast apoptosis, bone resorption	14,18	bleeding on probing, attachment loss
IL-6	Regulate bone remodeling	16	Stimulate osteoclast activity and bone resorption
Osteoprotegerin	Bone remodeling by inhibiting osteoclastogenesis	13,16	represent a defense mechanism against calcification, other forms of vascular damage, acting as an important regulatory molecule in the vasculature, association with calculus formation
AGE	burst in PMN release	16	Increase vascular damage
IL-1β	Facilitate leukocyte recruitment and stimulate the production of inflammatory mediators	17	Stimulate bone resorption and limit repair
sIgA	Interfere in bacterial adherence and bacterial metabolism	17	Edema and tissue permeability
Macrophage inflammatory protein 1α (MIP-1α)	Promote chemotaxis and transendothelial migration	19	indicate the presence of subclinical inflammation in periodontal clinically healthy sites
Hemoglobin	Oxygen transport	20	invisible bleeding in mild periodontal pockets when BOP is negative

SALIVA COLLECTION AND STORAGE METHODS

The ability to analyze salivary biomarkers to monitor health and disease is a highly desirable goal for oral health promotion and research. The successful measurement of salivary analytes requires the optimal time of collection, processing, and storage procedures. (21) Fasting saliva is preferred as certain components such as total protein, sodium, chloride, are influenced by circadian rhythm. However, the technique and materials used to collect samples may depend upon the specific constituents analyzed. (22) Saliva can be easily collected by passive drool (Table 4) directly into plastic tubes (unstimulated saliva), which is the most recommended procedure so far across all patients and within all collection visits for each individual.

Whole saliva collection method:

- Collect saliva from the subjects (please aim for 8–10 a.m. if possible) and ask the subject to refrain from eating, drinking, or oral hygiene procedures for at least 1 h prior to the collection.
- Give the subject distilled drinking water and ask that they rinse their mouth out well for 1 min. The subject can then expectorate or swallow the water.

- Five minutes after this oral rinse, ask the subject to spit into a 50 mL sterile tube. Encourage the subjects to place the tube on ice while collecting more saliva.
- Remind the subjects not to cough up mucus as the goal is to
- passively collect saliva, not phlegm
- Collect approximately 5 mL volume of saliva.
- Return to the laboratory immediately for processing. Processing should occur within a 1 h window of time.
- It is imperative that all saliva sample processing occurs as soon after collection as possible and that all samples remain on the ice at all times.

Processing:

Divide the saliva sample into multiple 330 µL samples placed in cryotubes able to accommodate 80◦C temperatures

Each 330 µL sample can now be further processed for storage according to the anticipated endpoint analysis, i.e., protein or RNA analysis.

(a) For samples intended for protein analysis add the following protease inhibitors to each 330 µL volume of saliva:

i. 0.33 µL aprotinin. Invert gently to mix.
ii. 1 µLNa3OV4(from standard stock of 400 mM). Invert gently to mix.
iii. 3.3 µL PMSF (standard stock of 10 mg/mL). Invert gently to mix.

(b) For samples intended for RNA analysis add the following RNase inhibitors to each 330 µL volume of saliva

i.65 µL(5µL SI/1 mL sample) SUPERase Inhibitor (Ambion).

For supernatant samples:

Some salivary diagnostic protocols may require supernatant saliva drawn from whole saliva fractions.

(1) Briefly vortex the whole saliva sample (fewer than 20 s) so that it whirls up the sides of the tube.

(2) Spin the entire sample at 2,600g for 15 min at 4◦C

(3) Remove the supernatant taking care not to disturb the "pellet" at the bottom of the tube and transfer the fractions to appropriately labeled cryotubes. Similarly, transfer the resultant pellet to a fresh tube.

(4) Add the RNase inhibitor and protease inhibitors to the supernatant fractions as described above.

Note: Do not add these reagents to the pellet.

(5) Store all fractions and the pellet at –80◦C.

Storage:

Store all fractions at –80◦C.

CONCLUSION

The current deficit in the development of diagnostic strategies favors a modern cultural approach for the diagnosis of oral diseases that identify cases of gingivitis that are at risk of progressing to periodontitis. Many investigations are carried out to detect the underlying disease. However, the development of disease biomarkers has not been fully realized due to an overall low concentration of these markers in saliva when compared to serum. Today, highly sensitive and high-throughput assays such as microarray, mass spectrometry, reverse transcriptase-polymerase chain reaction (RT-PCR), and nano-scale sensors can measure proteins and RNAs even at low concentrations in saliva, thus expanding the utility of saliva as a diagnostic fluid [23].

Advances in microfluidics technology like digital microfluidics appear promising to diagnose periodontal diseases by the use of a chair-side Lab-on-a-chip technology. These salivary biomarker detectors can be used by dentists for point-of-care disease screening and detection for monitoring the involvement of oral and systemic disorders.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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