Salivary proteomic biomarkers in the diagnosis of periodontal diseases

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Abstract

The biochemical analysis of saliva is becoming indispensable in dentistry. Monitoring salivary biomarkers for oral and systemic diseases could become an important complement to clinical examination. Current clinical diagnostic parameters that were introduced more than a century ago continue to function as the basic model for Periodontal diagnosis. Biomarkers, whether produced by normal healthy individuals or by individuals affected by specific systemic diseases are tell tale molecules that could be used to monitor health status, disease onset, treatment response and outcome. This review limits itself to proteomic analysis of saliva in the diagnosis of periodontal diseases.
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Introduction

Clinical diagnosis of periodontal disease is based on oral examination consisting of inspection of the gingival tissue, conducting a periodontal screening and recording pocket depths for each tooth, checking attachment level, measuring plaque index, testing bleeding on probing, testing tooth mobility & taking radiographs to assess bone loss. All these are informative to evaluate disease severity but provide few useful determinants of disease activity.\(^1\),\(^2\),\(^3\) Periodontal disease severity may be ascertained by the salivary level of periodontal pathogens / host response markers and the periodontopathic bacteria may be acquired from the infectious saliva of close family members.\(^4\),\(^5\),\(^6\) The periodontopathic bacterial species identified in whole saliva includes *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Treponema denticola*. Samples of both whole saliva & periodontal pockets may be needed to demonstrate/detect oral *A.actinomyetamcomitans* and *T. forsythia* as both of these can persist in non dental sites.\(^7\),\(^8\)

Umeda *et al* compared the presence of six species of periodontopathic bacteria in whole saliva and sub gingival plaque from 202 subjects. The organisms were more frequently present in whole saliva than in periodontal pockets. The study found a relationship between the presence of periodontopathic bacteria in whole saliva and in periodontal pockets.\(^9\),\(^10\)

Saliva can be easily collected, contains locally derived and systemically derived markers of periodontal disease and hence may offer the basis for a patient specific diagnostic test for periodontitis.\(^11\),\(^12\) Salivary analysis may offer a cost effective approach to assess periodontal disease incidence in large populations.\(^13\),\(^14\)

Recently there has been a growing interest in exploring the protein composition using advanced proteomics technology. The proteome is the protein complement of the genome and proteomics is analysis of the protein of the genome that is expressed. Human salivary proteomic analysis can provide information about the changes in the early stage of disease and monitor disease progression. Human salivary proteome analysis is important for understanding oral health and disease pathogenesis. Most relevant to periodontal disease are the emerging tool boxes of the salivary proteome and the salivary transcriptome for early detection, disease progression and therapeutic monitoring. Researchers have identified 1,1666 salivary proteins ,914 from the parotid fluid and 917 from the combined submandibular & sublingual fluids.\(^15\)

The various proteomic bio markers used for periodontal disease monitoring and treatment are discussed below.

Immunoglobulins

Immunoglobulins are important specific defence factors of saliva. Saliva contains secretory Ig A [S Ig A], IgG and Ig M.IgA2 is found in higher concentrations in tears, saliva and milk.\(^16\)

Patients with periodontal disease are shown to have higher salivary concentrations of
IgA, Ig G and IgM specific to periodontal pathogens compared with healthy patients.\textsuperscript{17-20} Saliva from treated periodontitis patients had higher IgA and IgG levels to \textit{Porphyromonas gingivalis} and \textit{T denticola} than as compared to saliva from controlled subjects. Increased concentrations of salivary IgG to \textit{A. actinomyetamcomitans} in patients of aggressive periodontitis was reported by Sandholm \textit{et al.}\textsuperscript{21}

**Enzymes**

Salivary esterase has been statistically associated with calculus formation. Salivary esterase levels were found to be useful in determining the efficacy of periodontal treatment. Lysozyme is an anti microbial enzyme with the ability to cleave chemical bonds in the bacterial cell wall. It may also cause lysis of bacterial cells by interacting with monovalent anions and with proteases found in saliva. Patients with low levels of lysozyme in saliva are more susceptible to plaque accumulation, which is considered a risk factor for periodontal diseases.\textsuperscript{22} Peroxidase is a salivary enzyme produced by acinar cells in the salivary glands. This enzyme removes toxic hydrogen peroxide produced by oral micro organisms and reduces acid production in the dental biofilm. Patients with periodontal disease have demonstrated high levels of this enzyme in saliva.\textsuperscript{23} Chitinase plays a role in the defence against chitin containing pathogens. Van steijn \textit{et al} reported that chitinase level was raised in the saliva of periodontitis patients which decreased on treatment.\textsuperscript{24}

Arginase is an arginine depleting enzyme. Significant variation in salivary arginase activity in periodontitis patients was reported by Ozmeric \textit{et al} in 2000.\textsuperscript{25} Beta glucuronidase in saliva is an indicator of neutrophil influx into gingival tissues and may provide as a risk factor in periodontal disease.\textsuperscript{26}

A significant positive correlation between salivary acid phosphates & calculus formation was observed by Draus \textit{et al.}\textsuperscript{27} It was found that mixed whole saliva of adult periodontitis patients revealed lowest enzyme activities. Other enzymes ,proteins and host response indicators in gingival crevicular fluid & saliva have been extensively explored using proteomic methods for their utility in periodontal disease diagnosis and include beta – galactosidase, beta and alpha glucosidase, caprylate esterelipa elastase, kininase, lactate dehydrogenase, myeloperoxidase, caprylate esterase, amino peptidase, aspartate aminotransferase, lactate dehydrogenase, cathepsin B , CD 14 , kallikrein.\textsuperscript{28-31} Nitric oxide ,which is a free radical with important cellular functions is produced and released from human neutrophils and macrophages. The increased salivary arginase activity causes a decrease in nitric oxide synthesis further leading to the decrease in the anti bacterial property of saliva, causing periodontal tissues to become more susceptible to existing pathogens.\textsuperscript{25}

**Salivary ion-calcium**

Calcium is the ion that has been most extensively studied as a periodontal marker for periodontal disease in saliva. A high concentration of calcium in saliva of periodontitis patients was observed by Sewon \textit{et al.}\textsuperscript{32}
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Proteins

Enzymes, proteins and immunoglobulin are the most abundant constituents of saliva. Recent studies have explored their value as biomarkers using proteomic methods.\textsuperscript{33,34,35} Mucins are glycoproteins produced by submandibular and sublingual glands and numerous minor salivary glands. The mucin MG2 affects bacterial aggregation and adherence and has a reverse relation with \textit{A. actinomycetemcomitans} colonization.\textsuperscript{36} Lactoferrin is an iron binding glycoprotein produced by salivary glands. An elevated level of lactoferrin is seen in saliva of subjects affected by periodontitis compared with healthy subjects.\textsuperscript{37} Histatin is a salivary protein. It is an inhibitor of host and bacterial enzymes involved in the destruction of the periodontium. Histatin also inhibits the release of histamine from mast cells.\textsuperscript{38,39} Fibronectin is a glycoprotein that mediates inflammation, wound healing and tissue repair. Cystatins are proteolytic enzymes having collagenolytic activity. They may function by modulating enzyme activity in the periodontium.

Aminoacids

Syrjanen \textit{et al} reported that elevated levels of certain aminoacids especially proline may be detected in periodontitis patients. This may be the result of bacterial metabolism/degradation of salivary proteins rich in proline. In contrast the same authors also reported no diagnostic significance of aminoacids in periodontal diseases.\textsuperscript{39}

Platelet activating factor/PAF

It is an apotent phospholipid mediator of inflammation. A positive correlation between PAF and periodontal inflammation has been reported by Garito \textit{et al} which is in agreement with similar studies but the potential diagnostic significance of their findings remain obscure.\textsuperscript{14,40}

Growth factors

The growth factors in GCF and saliva have been examined for their utility in periodontal disease diagnosis and include transforming growth factor alpha and transforming growth factor \(\beta\), platelet derived growth factor, vascular endothelial growth factor, hepatocyte growth factor and epidermal growth factor.\textsuperscript{41-46}

Epithelial keratins

It has been suggested that phenotypic markers for junctional and oral sulcular epithelia might be used as indicators of periodontal disease. No studies have shown a reliable association between number or type of epithelial cells /specific types of keratins in saliva and progression of periodontitis.\textsuperscript{14}

Hormones

Recently salivary cortisol levels were used to evaluate the role of emotional stress in periodontal disease. Higher salivary cortisol levels were detected in individuals exhibiting severe periodontitis.\textsuperscript{47}
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**Inflammatory cells**

The number of leukocytes in saliva varies from person to person and the cell counts vary for an individual during the course of the day. The majority of salivary leukocytes enter the oral cavity via the gingival crevice.\(^{48}\)

**Volatiles**

Volatile sulfur compounds, mainly H2S and methylmercaptan are associated with oral malodour. Salivary volatiles have been suggested as possible diagnostic markers and contributory factors in periodontal disease. Rosenberg *et al* observed that estimation of malodour based on saliva were significantly correlated with objective parameters.\(^{49}\)

**Markers in saliva via gingival cervicular fluid (GCF)**

GCF is both a physiological as well as an inflammatory exudate, originating from the gingival plexus of blood vessels in the gingival corium, subjacent to the epithelial lining lining of the dento gingival space. As GCF traverses through inflamed periodontal tissues *en route* to the sulcus, biological molecular markers are gathered from the surrounding areas and are subsequently eluted into whole saliva. Of the many constituents in GCF, however, the vast majority constitute soft tissue inflammatory events, while only a few are regarded as specific biomarkers of alveolar bone destruction.\(^{50}\)

**Markers of periodontal soft tissue inflammation**

Pro inflammatory cytokines, such as prostaglandin E 2[PGE 2];interleukin[IL-1 beta,IL-6 &TNF-alpha are released from cells of junctional epithelium, C 7 fibroblasts ,macrophages and PMN. CRP, C \(_3\),C \(_4\) and alpha -2M are humoral inflammatory markers and acute phase reactants. Decreased levels of these proteins in saliva of patients with chronic periodontitis indicate that host inflammatory response is decreased in chronic periodontitis.\(^{51,52}\)

IL-1\(\beta\) concentration in saliva from patients with periodontitis were slightly higher as seen in a recent study.\(^{53}\)

**TNF \(\alpha\)**

Salivary levels of TNF \(\alpha\) were significantly higher in individuals with periodontal disease than in controls. Subjects with salivary TNF alpha levels above a threshold of 5.75pg/ml had significantly more sites with bleeding on probing.\(^{54}\) Similar to these studies, Aurer *et al* had evaluated the pro inflammatory factors in saliva in periodontal diseases. The results were consistent with previous studies and they observed that C3 and alpha -2M were higher in Ag P in comparison to chronic periodontitis patients and edentulous patients.

**Markers of alveolar bone loss**

Many different biomarkers associated with bone formation, resorption and turnover such as alkaline phosphatase, osteocalcin,
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osteonectin and collagen telopeptidases have been evaluated in saliva and GCF. Matrix metalloproteinases/MMP

They are host proteinases responsible for both tissue degradation and remodeling. Host derived MMPs are considered to be key initiators of the extra cellular matrix degradation associated with periodontal disease. MMP 8 is a key enzyme in extra cellular collagen matrix degradation derived predominantly from PMNs during acute stages of periodontal disease. MMP 8 is the most prevalent MMP found in diseased periodontal tissue and GCF. MMP 8 and MMP 9 are also stored in neutrophilic granules. MMP 8 indicates both disease severity and disease activity. The level of MMP 8 was demonstrated to be highly elevated in saliva from patients with periodontal disease using a rapid point of care microfluidic device. The MMP 8 level is also elevated in peri implant sulcular fluid from peri implant lesions. The results of longitudinal study of patients with gingivitis, non progressive and progressive periodontitis illustrate elevated MMP 8 levels in active disease progression. The levels of MMP 8 correlated significantly with periodontal activity even after adjusting for the cofounders. The authors of a recent cross sectional study of salivary biomarkers demonstrated that combined levels of IL-1β and MMP-8 increased the risk of experiencing periodontal disease by 45 folds and elevation in all three biomarkers IL-1 beta, MMP-8 and osteoprotegerin correlated with individual clinical parameters that are indicative of periodontal disease. It was shown that the initiation of collagen degradation from the connective tissue and alveolar bone occurs due to an imbalance between MMPs and their inhibitors, tissue inhibitors of MMPs (TIMPs). TIMP-1 is an inhibitor of MMP-8. MMP-1 and TIMP1 are detected in the saliva of periodontitis patients but no significant elevated levels were observed. Higher levels of MMP -2 and MMP -3 were also reported in the saliva of patients affected by periodontitis. MMP-9 is a member of collagenase family which is produced by neutrophils degrades collagen intercellular ground substance. In their study Teng et al found a two fold increase in the mean MMP -9 levels in patients with progressive attachment loss. When investigators consider MMP 8/ MMP -9 with the red complex, they are better able to predict periodontal status. Red complex bacteria are known for their potent ability to produce the trypsin like enzyme activity that is responsible for destroying collagen matrices. MMP-13 is a collegenolytic MMP with an exceptionally wide substrate specificity. Ma et al demonstrated that elevated levels of both MMP -13 and MMP-8 correlated with irreversible perio-implant vertical bone loss around loosening dental implants.

Telopeptide

Carboxyterminal telopeptide of type I collagen has been shown to be a promising predictor of both future alveolar bone and attachment loss. Several investigations have explored the ability of pyridinoline cross links to detect bone resorption in
periodontitis and peri-implantitis as well as in response to periodontal therapy.  

**Alkaline Phosphatase**

Alkaline phosphatase is a membrane bound glycoprotein that is involved in maintenance of alveolar bone and renewal of the periodontal ligament. It was found that mixed whole saliva of adult periodontitis patients revealed the highest enzyme activities with alkaline phosphatase than that of healthy individuals who revealed lowest enzyme activities. This can be attributed to alveolar bone loss and hence it was concluded that salivary ALP could be used as a useful marker for monitoring periodontal disease.

**Osteocalcin**

It is a non-collagenous calcium binding protein synthesized mainly by osteoblasts. A number of investigators studied relationship between GCF osteocalcin levels and periodontal disease. When a combination of the biochemical markers osteocalcin, collagenase, prostaglandin-E2, alpha-2 macroglobulin, elastase and ALP was evaluated, increases diagnostic sensitivity and specificity values of 80% and 91% respectively were reported. Osteopontin is a single chain polypeptide with a molecular weight of approximately 32,600. Kido et al demonstrated that osteopontin level in GCF was increased with progression of periodontal disease. The osteopontin levels in GCF were significantly reduced when non-surgical periodontal treatment was provided. Calprotein, a major cytosol protein of leukocytes was demonstrated to be higher in GCF from periodontitis patients than healthy controls.

**Stress markers**

**C-reactive protein**

It is a systemic marker released during acute phase of an inflammatory response and is produced by the liver. Circulatory CRP reaches saliva via GCF/salivary glands. High levels of CRP are associated with chronic and aggressive periodontal diseases. C-reactive protein has recently been shown to be measurable in saliva from periodontal patients using a lab-on-a-chip method. The level of C-reactive protein is directly related to an individual’s periodontal status.

**Serum markers in saliva**

Researchers have identified emotional stress as a risk factor for periodontitis. It has been explained that the mechanism behind this is the elevated serum cortisol level during emotional stress that exert a strong inhibitory effect on the inflammatory process and immune response. Higher cortisol levels were detected in individuals exhibiting severe periodontitis and a high level of financial strain and high emotion as compared to individuals with little/no periodontal disease, low financial strain, and low levels of emotion.

**Oxidative stress marker**

Oxidative stress is enhanced during periodontitis. It has been demonstrated that the 8-OHdG (8-hydroxy deoxyguanosine) in bodily fluids can act as a...
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biomarker of oxidative stress. Studies have shown that saliva is a biological product that can be easily collected and may be used for the quantification of 8- OHdG as an oxidative stress bio marker in the diagnosis and monitoring of the treatment in periodontitis.77,78

Other markers

Salivary alaine amino transferase (ALT) and the counts of P.Gingivalis and the ratio between the two is currently being suggested as a potential indicator for the progression of periodontitis.79

Summary

To date, there is no single biomarker that is specific for periodontal disease. Therefore, there is strong potential for the use of microbial and host response biomarkers in combination to enhance identification of the disease process, given the multifactorial nature of periodontal disease.

It is envisioned that proteomic salivary tools can be used to identify markers for early detection, disease progression and therapy monitoring of patients with periodontal disease.

While many questions remain, the potential advantages of salivary analysis for the diagnosis of periodontal diseases suggest that further studies are warranted. Integrating these new salivary diagnostic methods into clinical practice is important to aid dental professionals in making essential health related decisions for patients.

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