TECHNIQUES

Salivary proteomic biomarkers in the diagnosis of periodontal diseases

TP Padma Kumar, MS Deepa *, K Nanda Kumar
Department of Periodontics, * Department of Oral Medicine
Azeezia College of Dental Sciences and Research, Kollam, Kerala

Correspondence to: padmakumartp@yahoo.com

Abstract

The biochemical analysis of saliva is becoming indispensable in dentistry. Monitoring salivary bio markers 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.

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.actinomycetamcomitans* 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 periodintitis. [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.

Imunoglobulins

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.[17-20] Saliva from treated periodontitis patients had higher IgA and IgG levels to *Porphyromonas gingivalis* and *T denticola* than as compared to saliva from controlled subjects.Increased concentrations of salivary IgG to *A. actinomycetamcomitans* in patients of aggressive periodontitis was reported by Sandholm *et al* .[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.[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.[23]Chitinase plays a role in the defence against chitin containing pathogens. Van steijn *et al* reported that chitinase level was raised in the saliva of periodontitis patients which decreased on treatment.[24]

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

A significant positive correlation between salivary acid phosphates & calculus formation was observed by Draus et al.[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.[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.[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 *et al*.[32]

Proteins

Enzymes, proteins and immunoglobulin are the most abundant constituents of saliva. Recent studies have explored their value as biomarkers using proteomic methods.[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 *A. actinomycetamcomitans* colonization.[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.[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.[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 *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.[39]

Platelet activating factor/PAF

It is apotent phospholipid mediator of inflammation. A positive correlation between PAF and periodontal inflammation has been reported by Garito *et al* which is in agreement with similar studies but the potential diagnostic significance of their findings remain obscure.[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 β, platelet derived growth factor, vascular endothelial growth factor, hepatocyte growth factor and epidermal growth factor.[41-46]

Epithelial keratins

It has been suggested that phenotypic markers for junctional and oral sulcular epethelia 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.[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.[47]

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-1β concentration in saliva from patients with periodontitis were slightly higher as seen in a recent study.[53]

TNF α

Salivary levels of TNF α 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,

osteonectin and collagen telopeptidases have been evaluated in saliva and GCF.[50]

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 .[55,56] 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.[57] 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.[58] The results of longitudinal study of patients with gingivitis, non progressive and progressive periodontitis illustrate elevated MMP 8 levels in active disease progression.[59,60] 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 I L-1 β and MMP- 8 increased the risk of experiencing periodontal disease by 45 folds and elevation in all three biomarkers IL- I beta , MMP-8 and osteoprotegerin correlated with individual clinical parameters that are indicative of periodontal disease.[61] 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.[53]

MMP-1 and T1MP1 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.[62] 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 specipicity. 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.[63]

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.[64]

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.[60]

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.[65,66,67] When a combination of the biochemical markersosteocalcin,collegenase, prostaglandin-E 2,alpha-2 macroglobulin, elastase and ALP was evaluated, increases diagnostic sensitivity and specificity values of 80% and 91% respectively were reported.[68] 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.[69] 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.[70]

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.[71] C – reactive protein has recently been shown to be measurable in saliva from periodontal patients using a lab-on – a chip method.[28]The level of C –reactive protein is directly related to an individual's periodontal status.[72]

Serum markers in saliva

Researchers have identified emotional stress as a risk factor for periodontitis.[73,74] 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.[75] 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.[76]

Oxidative stress marker

Oxidative stress is enhanced during periodontitis .It has been demonstrated that the 8-OHdG (8- hydroxyl de oxy guanosine) in bodily fluids can act as a

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.

References

1. Polson AM, Goodson JM. Periodontal diagnosis.Current status and future needs. J Periodontology 1985;56:25-34.

2. Armitage GC. Periodontal diseases:diagnosis. Am Periodontol 1996;1:37-215.

3. Greenstein G. Periodontal diseases are curable. J Periodontol 2002; 73:950-953.

4. Giannobile WV, BeiklerT, Kinney JS, Ramseier CA, Morelli T, Wong DT. Saliva as a diagnostic tool for periodontal disease:current state and future directions. Periodontol 2000-2001:50:52-64.

5. Paju S, Pussinan PJ, Suominan-Tiapale L, Hyvonen.M, Knuuttila M, Kononen E. Detection of multiple pathogenic species in saliva is associated with periodontal infection in adults. J Clin Microbiol 2009:47:235-238.

6. Asi Kainen S, Chen C, Slots J. Likelihood of transmitting Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in families with periodontitis. Oral Microbiol Immunol 1996; 1:387-394.

7. Emrani J, Chee W, Slots J. Bacterial colonization of oral implants from non dental sources. Clin Implant Dent Relat Res 2009;11:106-112.

8. Van Assche N, Van Essche M, Panwels M, Teughels W, Quirynen M. Do periodontopathogens disappear after full mouth tooth extraction? J Clin Periontol 2009;36: 1043-1047.

9. Umeda M, Chen C, Bakker I, Contreras A, Morisson JL et al. Risk indicators for harboring periodontal pathogens. J Periodontol 1998; 69 : 1111-1118.

10. Ummeda M, Contriras A, Chen C, Bakker I, Slots J. The utility of whole saliva to detect the oral presence of periodontopathic bacteria. J Periodontol 1998; 69: 828-833.

11. Aurer A, Srdjak KJ, Plancak D, Rukavina AS, Kozelk JA. Pro inflammatory factors in saliva as possible markers for PD. Coll Antropol 2005;29: 435-439.

12. Pezel J, Barig S, Bprso I, Abram M, Glazar I, Brumini G, Simunovic-Suskic M. Mediators. Inflamm 2004;13:131-5.

13. Oshima M, Fujikawa K, Kutagawah a, Ito K, Otsuka K. Hepatocyte growth factor in saliva. J Oral science 2002;44:35-39.

14. Kaufman E, Lamster IB. Analysis of saliva for periodontal diagnosis-a review. J Clin.Periodontol 2000;27 : 453-465.

15. Denny P, Hagen FK, Hardt M, Liao L, Yan W, Arellanno M. The proteomes of human parotid and sub mandibular/sublingual gland saliva collected as the ductal secretions. J Proteome Res 2008; 7:1994-2006.

16. Declacroix DL, Dire C, Rambaud JC, Vaerman JP. Ig A subclasses in various secretions and in serum. Immunology 1982;47:383-5.

17. Seemann R , Hagewald SJ, Sztankay V, Drews J, Bizhang M, Kage A. Levels of parotid and submandibular/sublingual salivary immunoglobulin A in response to experimental gingivitis in humans. Clin Oral Investig 2004;8:233-237.

18. Bachrach GMuster Z, Raz I, Chaushu G, Stabholz A, Nussbaum G, Gutner M, Chaushu S. Assessing the levels of immunoglobulins in the saliva of diabetic individuals with periodontitis using checkerboard immunodetection. Oral Dis 2008;14:51-59.

19. Guven O, De Visscher JG. Salivary IgA in PD. J Periodontol 1982;53:334-5.

20. Sandholm L, Grenblad E. Salivary immunoglobulins in patients with juvenile periodontitis and their healthy siblings. J Periodontol 1984;55:19-12.

21. Sandholm L, Tolo E, Olsen I. Salivary Ig G, a parameter of PD

activity? High responders to Actinobacillus actinomycetamcomitans in juvenile and adult periodontitis. J Clin Periodontol 1987;14:289-94.

22. Jalil RA, Ashley FP, Wilson RF, WagaiyuEG. Concentrations of thiocyanate, hypo thiocyanite, "free" and "total"l ysozyme, lactoferrin and secretory Ig A in resting and stimulated whole saliva of children aged 12-14 years and the relationship with plaque accumulation and gingivitis. J Periodont Res 1993;28:130-6.

23. Guven Y Satman I , Dinccag N, Alptekins. Salivary peroxidase activity in whole saliva of patients with type I diabetes mellitus. J Clin Periodontol 1996;23:879-81.

24. Van Steijn GJ, Amerongen AV,Veerman EC, Kasanmoentalib S, Overdijk B. Effect of periodontal treatment on the activity of chitinase in whole saliva of periodontitis patients. J of Periodontal Research 2002;37:245-9.

25. Ozmeric N, Elgun S, Uraz A. Salivary arginasein patients with adult periodontitis. Clinical Oral Investigation 2000;4:21-4.

26. Lamster IB, Kaufman E, Grbic JT, Winsto LJ, Singer RE. B –glucoronidase activity in saliva:relationship to clinical periodontal parameters. J of Periodontology 2003;74:353-9.

27. Draus FJ, Tarbet WJ, Miklos FL. Salivary enzymes & calculus formation. J of Periodontal Res 1968;3232-235.

28. Zia A, Khan S, Bey A, Gupta ND, Mukhtar-Un-Nisar S. Oral biomarkers in the diagnosis & prognosis of PD. Biology & Medicine 2011; 3:45-52.

29. Khashu H, Baiju CS, Bansal SR, Chhillar A. Salivary biomarkers : A Periodontal overview. J of Oral Health and Community Dentistry 2012;6:28-33.

30. Zhang L, Henson BS, Paulo M, Carmago, Wong DT. The clinical value of salivary biomarkers for PD. Periodontology 2000 2009;51:25-37.

31. Malamud D. Saliva as a diagnostic fluid. Dent Clin N Am 2011;55:159-178.

32. Seppa O. Calcium and other salivary factors in periodontitis affected subjects prior to treatment. J Clin Periodontol 1995;22:267-70.

33. Goannobile WV, Al-Shamari KF, Sarment DP. Matrix molecules and growth factors as indicators of PD activity. Periodontol 2000 2003; 31: 125-134.

34. Kinney JS, Ramseier CA Giannobile WV. Oral fluid –based biomarkers of alveolar bone loss in periodontitis.

Ann NY Acad Sci 2007;1098:230-251.

35. Ozmeric N. Advances in Periodontal disease markers. Clin Chim. Acta 2004;343:1-16.

36. Giannobile WV, Beikler T, Kinney JS, Ram Seier CA, Morelli T, Wong DT. Saliva as adiagnostic tool for PD: Current state and future directions. Periodontol 2000 2009;50:52-64.

37. Groenink J, Walgreen-Weterings E, Nazmi K, Bolscher JG, Veerman EC, Van Winkelhoff AJ, et al. Salivary lactoferrin low – Mr mucin MG2 in Actinobacillus actinomycetamcomitans associated periodontitis. J Clin Periodontol 1999;26:269-275..

38. Helmerhorst EJ, Oppenhein FG. Saliva: A dynamic proteome. J Dent Res 2007;86:680-93.

39. Syrjanen SO, Alakuijala H. Free AA levels in oral fluid of normal subjects and patients with PD. Arch Oral Biol 1990; 35:189-93.

40. Garito ML, Prihoda TJ, Mc Manus LM. Salivary PAF levels correlate with the severity of periodontal inflammation. J Dental Res 1995; 74:1048-56.

41. Mogi M, Otogoto J, Ota N, Inagaki H, Minami M, Kojima K, et al. Interleukin 1β, interleukin 6, B 2-microglobulin and transforming factor α in GCF from human PD. Arch Oral Biol 1999;44:535-539.

42. Salcetti JM, Moriarty JD, Cooper LF, Smith FW, Collins JG, Socransky SS, Offenbacher S. The clinical, microbial and host response characteristics of the failing implant. Int J Oral Maxillofac Implants 1997; 12: 32-42.

43. Skaleric U, Kramar B, Petelin M, Pavlica Z, Wahl SM. Changes in TGF β1 levels in gingival crevicular fluid and serum associated with periodontal inflammation in humans and dogs. Eur J Oral Sci 1997;105:136-142.

44. Booth V,Young S, Cruchley A, Taichman NS, Paleolog E. Vascular endothelial growth factor in human PD. J Periodontal Res 1998;33:491-499.

45. Taichman NS, Cruchley AT, Fletcher LM, Hagi-Pavli EP, Paleolog EM, Abrams WR, et al.Vascular endothelial growth factor in human salivary glands and saliva: a possible role in the maintenance of mucosal homeostasis. Lab Invest 1998;78:869-875.

46. Hormia M, Thesleff I, Perheentupta J, Pesonen K, Saxen L. Increased rate of salivary epidermal growth factor secretion in patients with juvenile periodontitis. Scand J Dent Res 1993; 101 :138-144.

47. Genco RJ, Hoa AW, Kopman , Grossi SG, Dunford RG,Tedesco LA. Models to evaluate the role of stress in PD. Ann Periodontol 1998;3:288-302.

48. Schiott RC, Loe RH. The origin and variation in number of leukocytes in the human saliva. J Periodont Res 1970; 5:36-41.

49. Rosenberg M, Kozlovsky A, Gelernter I, Cherniak O, Gabbay J, Bant R, et al. Self estimation of oral malodour. J Dent Res 1995; 74:1577-82.

50. Kinney JS, Ramseier CA, Giannobile WV. Oral fluid based biomarkers of alveolar bone loss in periodontitis. Ann N Y Acad Sci 2007;1098:230-251.

51. Brock G, Butterworth CJ, Mathews JB, Chapple ILJ. J Clin Periodontol 2004;31:515-518.

52. Sculley DV, Langley-Evans SC. Proc Nutr Soc 2002; 61:137-19.

53. Rathnayake N, Akerman S, Klinge B, Lundegren N, Jansson H, Tryselius Y, et al. Salivary biomarkers of oral health- a cross-sectional study. J Clin Periodontol 2013; 40:140-147.

54. Thomas MV, Miller CS. Bone remodeling biomarkers of PD in saliva. J Periodontol 2008; 79:1913-1919.

55. Sorsa T, Suomalainen K, Uitto VJ. The role of GCF and salivary interstitial collagenases in human PD. Arch Oral Biol 1990; 35:1935-1965.

56. Uitto VJ, Suomalainen K, Sorsa T. Salivary collagenase –origin , characteristics & relationships to periodontal health. J Periodontal Res 1990;25:135-142.

57. Herr AE, Hath AV, Throckmorton DJ, Tran HM, Brennan JS, Giannobile WV, et al. Microfluidic immune assays as rapid saliva based clinical diagnostics. Proceedings of National Academy of sciences USA 2007;104:5268-5273.

58. Kivela-Rajamaki , Maisi P, Srinivas R, Tervahartiala T. Levels and molecular forms of MMP-7and MMP-8 in diseased human peri – implant sulcular fluid. J Periodontal Res 2003; 38:583-590.

59. Birkedal – Hansen H. Role of matrix metalloproteinases in human PD. J Periodontol 1993;64:474-484.

60. Miller CS, King CP Jr, Langub MC, Kryscio RJ, Thomas MV. Salivary biomarkers of existing PD: a cross sectional study. J A M Dent Assoc 2006; 137:322-329.

61. Kathariya R, Pradeep AR. Salivary proteomic biomarkers for oral diseases. A review of literature.

Archives of Oral Sciences & Research 2010;1(1):43-49.

62. Teng YT, Sodek J, McCulloch CA. GCF gelatinase and its relationship to PD in human subjects. J Periodontol Res 1992; 27:544-52.

63. Ma J, Kitti U, Teronen O, Sorsa T, Husa V, Laine P, et al. Collagenases in different categories of peri-implant vertical bone loss. J Dent Res 2000; 79:1870-1873.

64. Oringer RJ, Palys MD, Iranmanesh A, Fiorellini JP, Haffajee AP, Socransky SS, et al. C-telopeptide pyridinoline cross links and periodontal pathogens associated with endosseous oral implants. Clin Oral Implants Res 1998;9:365-373.

65. Kunimatsu K, Mataki S, Tanaka H, Mine N, Kiyoki M, Hasoda K, et al. A cross –sectional study on osteocalcin levels in GCF from periodontal patients. J of Periodontology 1993;64:865-869.

66. Nakashima K, Roehrich N, Cimasoni G. Osteocalcin, prostaglandin E2 and alkaline phosphatase in GCF :their relations to periodontal status. J of Clinical Periodontology 1994; 21: 327-333.

67. Golub LM, Lee HM, Greenwald RA, Ryan ME, Sora T. A matrix metalloproteinase inhibitor reduces bone type collagen degradation fragments and specific collagenases in GCF during adult periodontitis . Inflammation Research 1997;46:310-319.

68. Nakashima K, Giannopaulo C, Anderson E, Roehrich N, Brochut P, et al. A longitudinal study of various crevicular fluid components as markers of PD activity. J Clin Periodontol 1996;28:832-8.

69. Kohri K, Nagata T. Osteopontin in GCF. Journal of Periodontal Research 2001;36:328-33.

70. Kido J , Nakamura T , Kido R ,Ohishi K, Yamauchi V, Kataoka M, Nagota T, et al. Calprotectin in GCF correlates with clinical and biochemical markers of PD. J of Periodontology 1999; 26: 653-7.

71. D- Aiuto F, Ready D ,Tonetti MS . Periodontal disease and c-reactive protein –associated cardiovascular risk. J of Periodontal Research 2004;39:236-241.

72. Pederson ED, Stanke SR, Whitener SJ, Sebastiani PT, Lambarts BL, Turner DW, et al. Salivary levels of alpha -2 macroglobulin ,alpha 1, antitrypsin,C-reactive protein, cathespin G & elastase in humans with /without destructive periodontal disease. Arch Oral Biol 1995;40:1151-1155.

73. Genco RJ. Current view of risk factors for periodontal diagnosis : A review. J Clin Periodontol 2000;27:453-65.

74. Linden GJ, Mullally BH, Freeman R. Stress and the progression of periodontal diseases. J of Clin Periodontol 1996;23:675-80.

75. Chrousos GP, Gold PW. The concepts of stress and stress system disorders: Overview of physical and behavioral homeostasis. J Am Med Assoc 1992;267:1244-52.

76. Sculley DV, Langley –Evans SC. Periodontal disease is associated with lower antioxidant capacity in whole saliva with evidence of increased protein oxidation. Clin Sci 2003; 105: 167-72.

77. Badea V, Balaban D, Amariei C, Nuca C, Bucur L. Salivary 8-hydroxy-2-deoxy guanosine as an oxidative stress marker for the diagnosis of Periodontal diseases. Farmacia 2010;58:5.

78. Patil PB, Patil BR. Saliva : A diagnostic biomarker of periodontal diseases. Journal of Indian Society of Periodontology 2011; 15:310-317.

79. Yoshiaki Nomura, Yasuko Shimada, Nobuhiro Hanada, Yukihiro Numabe, Kyuichi Kamoi, Tsutomu Sato, et al. Salivary biomarkers for predicting the progression of chronic periodontitis. Archives of Oral Biology 2012; 57:413-420.