U.PORTO
FACULDADE DE
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Salivary proteins as biomarkers for periodontal disease

Artigo de revisão bibliográfica

Mestrado Integrado em Medicina Dentária

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2012

Índice

Abstract

Recent research are directing the diagnostic methods for periodontitis disease so that the activity can be identified by quantifying biomarkers found in samples of gingival crevicular fluid, serum and saliva.

Periodontitis is an inflammatory disease that affects the connective tissue insertion and the supporting bone around the tooth. The triggering of the disease stimulates the production of inflammatory proteins which can be reflected in the saliva.

Saliva is a physiological fluid that is produced by salivary glands. It has a complex composition containing locally secreted proteins, as well as molecules of the systemic circulation. These characteristics make it a potentially good candidate to identify biomarkers that reflect changes resulting from periodontitis and other systemic diseases.

Due to that, this review article aims to compile the existing knowledge in this area, based on relevant literature about this subject and to analyze the advances that have been made in the field, and to clarify if the biomarkers present in saliva constitute a valid alternative in the diagnosis.

Resumo

Pesquisas recentes estão a direccionar os métodos de diagnóstico da Periodontite para que a actividade da doença possa ser identificada através da quantificação de biomarcadores encontrados em amostras de fluido crevicular gengival, soro e salíva.

A periodontite é uma doença inflamatória que afeta o tecido conjuntivo de inserção e o osso de suporte a volta do dente. O desencadear desta doença estimula a produção de proteínas inflamatórias que pode ser reflectida na saliva.

A saliva é um fluido fisiológico, produzido nas glândulas salivares. Tem uma composição complexa, contendo proteínas secretadas localmente, bem como moléculas de circulação sistémica. Estas características fazem com que seja um potencialmente bom candidato para identificar biomarcadores que reflitam alterações decorrentes da periodontite e de outras doenças bocais e sistémicas.

Deste modo, este artigo de revisão tem como objetivo compilar o conhecimento existente nesta área, baseado em literatura relevante sobre este tema dando a conhecer os avanços que têm sido feitos assim como esclarecer se atualmente os biomarcadores presentes na saliva constituem uma alternativa válida no diagnóstico.

Key words

Periodontitis, Saliva, Biomarkers, inflammation mediators, diagnosis

Introduction

Peridontal disease is the leading cause of tooth loss in adults[1] Periodontitis is an inflammatory condition initiated by the pathogenic bacterial biofilm of the tooth supporting tissues[2]. Nevertheless, it is the activation and mediation of host inflammatory responses that are ultimately responsible for the destructive events occurring in the periodontum. Infact, studies of the host immune response to pathogenic bacteria have contributed to the present understanding of the pathogenesis of periodontal diseases[3 4 5].

Periodontal pathogens activate host cells to produce proinflammatory mediators and enzymes, which in turn promote the destruction of periodontal tissues. Stimulation of epithelial cells, neutrophils, macro phages, and lymphocytes, with microbial components, increases cellular secretion of inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-a (TNF-α) . Also, the release of lysosomal and cytoplasmic enzymes, like for example, neutrophil-based elastase, myeloperoxidase, matrix metalloproteinases (MMPs), and lactate dehydrogenase (LDH), into periodontal tissues is higher in the areas with inflammation[67 8].

Diagnostic methods used in clinical practice today lack the ability to detect the onset of inflammation and to identify those patients who are susceptible to future disease progression[9].

In fact the traditional diagnosis of periodontitis is based on clinical and radiographic assessments of periodontal tissues . Clinical parameters are employed such as probing depth (PD), bleeding on probing (BP), clinical attachment levels (CAL) , plaque index (PI), and radiographic loss of alveolar bone [10]

However, the most desirable goal in health care promotion, and delivery would be to monitor the periodontal health status and the onset, progression, and treatment outcome of periodontal diseases through non-invasive means.

As an easily collected and non-invasive specimen, saliva has been used as a diagnostic fluid in medicine [11] [12]

In this context, it appears as a potentially useful diagnostic tool due to its easy collection and presence of locally-derived and systematically-derived markers of periodontal disease[4] [5]

For periodontal disease assessment, most of the research has focused primarily on gingival crevicular fluid (GCF) biomarkers that provide local disease status, but it represents a difficult approach to use for clinical applications [13] [14]. Although GCF is considered as the main source of periodontitis-associated cytokines and host- derived enzymes , these agents will eventually pass to saliva, which, unlike GCF, appears as an easy and non-invasive collectible specimen for periodontal disease detection . The major aim in the research of salivary diagnosis of periodontal disease is to find marker(s) that could be used, preferably as chair-side tests, for example, to determine the extent of periodontitis or the results of periodontal treatment, or even to a lesser extent to detect periodontitis in field studies .[15]

Within this context, this review aims to compile the existing knowledge in this area, describing the progress that has been done and analyzing if biomarkers present in saliva constitute a potentially valid alternative in periodontal disease diagnosis.

Material and methods

To carry out this review, it was made a research on the topic "salivary proteins as biomarkers for periodontal disease". The articles used were published between 2005-2012 in EBSCO Host databases (CINAHL, Pre-CINAHL, MEDLINE, BIOMED, PUBMED) and B-ON (Academic search premier Elsevier, Springer Link and Wiley intercience) as well as in journals printed on paper, available in the FMDUP library, using the combination of the following keywords: Periodontitis, Saliva, Biomarkers, inflammation mediators, diagnosis.

This search retrieved about 160 articles of which 67 articles were used. The exclusion criteria were:

- Scientific articles published before 2005
- Articles with incomplete abstract or full text not available

Pathogenesis of periodontal disease

Subgingival bacterial biofilm (plaque) initiates gingival inflammation (gingivitis). The first bacteria that form a biofilm on cleaned tooth surfaces are from the Streptococci species (gram-positive cocci). Thus supragengival plaq ue is firstly formed by a colonization of gram-positive organisms. Within two days gram-negative bacteria also appear in the subgingival biofilm and gingival inflammation begins [17]. At this point, gram-negative organisms are no longer subjected to normal oral hygiene procedures. Endotoxins (lipopolysaccharides present in the cell wall of gram-negative organisms released during cell death) are an important cause of gingival tissue damage caused by Gram negative bacteria in the subgingival biofilm[1617]. Endotoxins directly destroy gingival cells. They enhance tissue destruction due to stimulation of the immune response associated with gingival inflammation. Tissue damage is promoted through cytotoxicity, complement activation, and bone resorption. [17] Bacteria first contact with immune system occurs in epithelium with Langerhams cells which process antigens[1617].

Bacterial products that contact gingival connective tissue initiates an acute inflammatory response leading to vasodilatation, edema and polymorphonuclear leukocyte (P.M.N) activation [16]. One of the first effects of bacterial contact with gingival tissue is activation of mast cells. Mast cells have Toll like receptors which initiate production of vasoactive substances such as histamine which induce vascular permeability and vasodilatation[17]. This vasodilatation and increased permeability of capillaries is associated with edema and diapedesis of leukocytes from the blood vessels into the connective tissue of the gingiva. Mast cells produce other inflammatory mediators such as Slow- Reacting Substance of Anaphylaxis, leukotriene C4, Tumor Necrosis factor alpha (TNF-α).and IL-6. [17]These activate the acute inflammatory response. Polymorphonuclear leukocytes (P.M.N.s) are the characteristic dominant inflammatory cells of acute inflammation of the gingival. PMNs are able to migrate through tissue towards chemotaxic substances such as peptides from bacteria, saliva, Endotoxin,IL8 and activated complement. PMNs are able to engulf bacteria by a process of phagocytosis.[17]

Monocytes become macrophages in inflamed gingival tissue. They produce cytokines such as IL-1 and TNF-α which have a wide inflammatory influence as well as stimulating osteoclasts proliferation, differentiation and activation [17]. PGE2 another activator of inflammation and osteoclastogenisis, is also secreted in response to IL-1,TNF-α and LPS.[16 17]

As gingivitis develops the initial acute inflammatory response continues and a chronic inflammatory response is developed. Lymphocytes and capillary proliferation are two important factors that characterize this chronic inflammation[17].

Activation of complement is another part of the immune response seen in gingivitis [16]. Activated T lymphocytes produce cytokines that enhance inflammation and can also cause damage to gingival and periodontal cells. Two important cytokines are iIL-1 and TNF-α. The combined acute and chronic inflammation observed in gingivitis and periodontitis is destructive to bacteria. However, it also causes damage to the connective tissue of gingival and the periodontal tissues [17]. Another mechanism associated with the breakdown of connective tissue in periodontal disease involves matrix metalloproteinases (M.M.P). These are produced by PMNs, Macrophages ,Fibroblasts and Epithelial cells [17]. Cytokines such as IL-1β induce MMP production from fibroblasts, wich is inhibited by TIMPs and α2 macroglobulin. This equilibrium that regulate the production of MMP can be changed by inflammation. [17]

Bone destruction is a consequence of osteoblastsic and/or inflammatory cells activation of osteoclast differentiation via the production of cytokines such asM-CSF, RANKL, IL-1, TNF-α and Prostaglandins (PGE)[17]. Activated osteoclasts destroy the inorganic and organic bone components by the release of acid.and lytic enzimes, such as cathepsin k[17]. MMPs from osteoblasts will also destroy the organic collagenous components of the bone. [17]

Thus, the bacterial-induced inflammation on gingivitis can spread apically and involves the destruction of connective tissue of the periodontum, including bone[17]. This results in periodontal disease. The damaged epithelium of the gingival sulcus proliferates apically. Since loss of attachment of connective tissue fibers to cementum occurs, the pocket epithelium migrates to line the root surface[17].

Auxiliary exams in periodontitis diagnostic

Clinical exams

Probing depth - periodontal probing is the best diagnostic tool to gather information regarding the health status and attachment level of periodontal tissues. It serves multiple purposes: to assess the hemorrhagic response to physical pressure; to determine the presence of etiologic factors such as calculus, defective dental restorations, and root erosion; to locate the cement-enamel junction (CEJ); and to determine the pocket dimensions[18]. Periodontal probing has several drawbacks when used to monitor periodontal status longitudinally. Despite its lack of accuracy in determining sulcus or pocket depth, probing provides the clinician with a useful estimate of the location of the coronal insertion of intact connective-tissue fibers into the root[18].

Bleeding on probing - The bleeding on probing (BOP) is a widely used clinical sign as indicator of the periodontal condition and disease progression. BOP as predictor of future periodontal deterioration seems to significantly increase when associated with periodontal pocket depth greater than or equal to 6 mm[19].

Clinical attachment levels – Attachment levels are excellent indicators of past destruction of the periodontal attachment apparatus and can be used to monitor the progression of periodontitis[20]. Attachment level measurements are more frequently used as clinical end-points in clinical trials than by private practitioners to determine the periodontal status of patients and to monitor patient responses to periodontal therapy. Clinical attachment level measurements have been used in clinical trials to evaluate a systemic host modulatory agent, demonstrating their utility as surrogate markers of efficacy [20]

Plaque index – The quantification of the amount and distribution of plaque on tooth surfaces, assesses the thickness of plaque at the cervical margin of the tooth (closest to the gum). Four areas, distal, facial or buccal, mesial, and lingual, are examined. Each tooth is dried and examined visually using a mirror, an explorer, and adequate light. The explorer is passed over the cervical third to test for the presence of plaque.[21]

Radiographic loss of alveolar bone – Radiographs are used to assess severity and pattern of bone loss, root length, anatomy and position, and to detect pathologic lesions[22]. Signs such as enlargement of the periodontal ligament space, absence of the lamina dura, bone defects (vertical and horizontal) and a diffusion image in the furcation area, associated with clinical signs, are suggestive of the presence of periodontal disease.[22] . Radiographs taken from proximal sites suggestive of mild bone loss, can be used to control the progression of the defect years later [23].

Laboratory exams

Enzyme-Linked Immunoabsorbent Assay (ELISA) – The purpose of an ELISA assay is to determine if a particular antigen (usually a protein) is present in a sample and, if so, how much. ELISA separates some component of the analytical reaction mixture by adsorbing them onto a solid phase which is physically immobilized. In the ELISA assay a liquid sample is added onto a stationary solid phase with special binding properties. After some incubations and washes with multiple liquid reagents that are sequentially added, it occurs some optical change in the final solution in the well from which the quantity of the analyte is measured. For that, an enzyme reacts with the appropriate substrates wish elicits a change in the color of the solution occurs, which is used as a signal. However, the signal has to be associated with the presence of an antibody or antigen, which is why the enzyme has to be linked to an appropriate antibody[24]

Time-resolved immunofluorometric assays (TR- IFMA) - The assay is based on a system that embodies a dry-reagent all-in-one immunoassay concept and utilizes a stable fluorescent lanthanide chelate as well as time-resolved fluorometry[25]. TR-IFMA is characterized by lower detection limits and greater specificity, reproducibility and practicability[25]. Ultrasensitive immunoassay methods are developed and used in clinical diagnostics to measure extremely low concentrations of specific compounds in highly complex samples [25].

Possible salivary biomarkers

Inflammatory biomarkers

Periodontal disease initiates with inflammation of the gingival apparatus and periodontal tissues in response to bacterial plaque accumulation. The persistent presence of the multispecies bacterial biofilm leads to chronic inflammation and an abundance of inflammatory molecules in oral fluids, that can be monitored and used as biomarkers for the disease.

Interleukine-1β - (IL-1β) - Interleukine-1β is a pro-inflammatory cytokine that induces widespread gene expression, including cyclo-oxygenase-2, inducible nitric oxide synthetase, and metalloproteinases that can contribute to the activation of osteoclasts and, consequently, to the bone resorption.[26] This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). It is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis [27]. Of the two isoforms of IL-1 (i.e., IL-1α and IL-1β), IL-1β is more potent in stimulating bone resorption and is the more most frequently occurring form in periodontitis.[28] It is produced during periodontal inflammation and tissue destruction[29] In the periodontum, IL-1β may be synthesized and secreted by the local connective tissue cells (fibroblasts and endothelial cells), or by the infiltrating leukocytes In clinical studies, increased levels of IL-1β have been detected in GCF and have been associated with gingival inflammation, periodontal disease severity and an absence of therapeutic effectiveness.[30] The levels of IL-1β also positively correlated with several periodontal indices parameters: bleeding on probing, clinical attachment level, percentage of sites with pocket depths of at least 4 mm and overall periodontal disease severity.[29]

Interleukine-6 (IL-6)- IL-6 displays a range of functions, including acute-phase protein induction, B- and T-cell growth and differentiation, and plays a crucial role in osteoclast generation and activation. [32] IL-6 is produced by T and B cells, macrophages, endothelial cells, epithelial cells and fibroblasts in response to infection, stress and neoplasia. It is also released in response to IL-1 and TNF stimulation of many of these cell types[32] It is responsible for the transition from acute to chronic inflammation by modi-

fying the present leukocyte infiltration (from polymorphonuclear neutrophil to monocytes/macrophages) and by stimulating the T and B cells, favoring chronic inflammatory response [31]. Furthermore, it activate cellular differentiation of mesenchymal stem cells for the osteoblastic lineage, it is a potent anti-apoptotic agent in osteoblastic cells, being therefore, responsible for bone resorption and inducing the production of metalloproteinasese [32]. Costa et all e Ng PY et all showed in their studies that, IL-6 levels were directly proportional to bone loss scores of adult patients with chronic periodontitis[29 33]. Thus, IL-6 appears to be a useful indicator or marker for diagnosis of periodontitis [33].

Tumor necrosis factor- α (TNF-α) – TNF- α is a proinflammatory and immuno-regulatory cytokine central to the pathogenesis of various inflammatory conditions [34]. It plays a role in the recruitment of inflammatory cells and bone resorption through its ability to stimulate IL-1 and granulocyte macrophage colony-stimulating factor (GM-CSF) expression, to inhibit bone collagen synthesis, to induce collagenases expressions and by stimulating osteoclast differentiation in the presence of GM-CSF. Although one report suggested that TNF-α was difficult to detect in saliva 39, others found low levels of TNF-α in that fluid [29 30]. Frodge et all reported that TNF-α levels were detectable in all salivary samples from[3536] patients, who had chronic adult periodontal disease and in healthy the controls [36]. In addition, they found that TNF-α levels in saliva were significantly elevated (by two times) in periodontitis subjects compared with the controls. As it happens with IL-1β, elevated salivary TNF-α levels correlated with an increased number of sites with bleeding on probing, pocket depth sites of at least 4 mm and clinical attachment levels of at least 2 mm [3.] Similar findings have been reported by Ng *et al*[29]. These data suggests that salivary TNF-α levels may have utility for the screening diagnosis of chronic periodontitis in adults.

Macrophage inflammatory protein (MIP)-1α - (MIP)-1α is a member of the cysteine-cysteine chemokine family, which is secreted by inflammatory cells and is primarily associated with cell adhesion and migration[37]. It stimulates monocytes and/or osteoclast progenitor cells tobecome active osteoclasts in a dose-dependent manner [37]. It is a chemokines crucial for Immune responses during infection and inflammation, being produced by macrophages after they are stimulated with bacterial endotoxins. It activates human granulocytes (neutrophils, eosinophils and basophils) which can lead to

acute neutrophilic inflammation[37]. It also induce the synthesis and release of other pro-inflammatory cytokines, such as interleukin 1 (IL-1), IL-6 and TNF-α, from fibroblasts and macrophages [38]. MIP-1α has been detected at higher levels in saliva in a longitudinal study of adolescents who had aggressive periodontitis compared with controls [24]. However, MIP-1α does not seem to be elevated in the saliva of chronic adult-periodontitis patients [29]

Molecules of connective tissue destruction

The destruction of connective tissue matrix is responsible for the pathogenesis of chronic inflammatory states, such as periodontitis [17]. Degradation of the matrix is initiated extra- and pericellularly by proteinases produced locally at the inflammatory site and balanced by inhibitors of proteinases. The level of balance/imbalance is thought to determine the progression rate of chronic periodontitis.

Matrix metalloproteinase-8 (MMP-8) - Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes that degrade the extracellular collagen matrix and are involved in the healing of injured tissue[39]. They are predominantly derived from polymorphonuclear leukocytes. Thus, their expression levels are low in noninflamed periodontium but are significantly higher at sites of periodontal inflammation, being related to the migration of polymorpho-nuclear leukocytes into diseased sites. [39] MMP-8 is the main destructive metalloproteinase (MMP), involved in the pathogenesis of periodontitis and is found in high amounts in gum biopsy and in oral fluids .[40] . MMP-8 has the unique ability to break down type I and III collagens, which are the major collagen species within the periodontium. MMP-8 also acts in a protective/anti-inflammatory manner, inhibiting alveolar bone loss in a murine model of bacterium-induced periodontal disease.[41]. MMP-8 has been readily detected in the saliva of patients experiencing periodontal disease[424344]. In fact salivary MMP-8 levels are elevated in patients with aggressive periodontitis compared with healthy controls[25]. Elevated salivary MMP-8 levels correlated highly with clinical measures consistent with the features of periodontal disease, and elevated levels of MMP-8 were significantly associated with an increased risk for clinical parameters of periodontal disease. It was also demonstrated that both MMP-8 and MMP-9 levels were detectable in the saliva of patients with chronic periodontitis and levels of MMP-8, but not MMP-

9, decreased after conventional therapy (i.e., scaling and root planning) as well as after doxy cycline therapy [25] So, it suggests that MMP-8 has an important role in monitoring of periodontitis development.

Aminotransferases - Aminotransferases (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) are enzymes relevant to periodontal disease diagnosis[46]. Both are cytoplasmic enzymes important for the metabolism of various amino acids and serve as diagnostic analytes of cellular injury in clinical chemistry[4546]. AST and ALT catalyze amino-transfer or transamination, during the carabolic and anabolic pathways of aminoacids. They are ubiquitous components of saliva and are detected in periodontal tissue, GCF [46], the enamel pellicle [47] and saliva. Fibroblasts from the periodontal ligament produce significantly lower levels of aminotransferases than gingival epithelial cells. In one study of patients with periodontal disease, salivary AST activity was significantly increased (by five times) compared with controls, whereas salivary ALT activity was not significantly altered in patients with periodontal disease[31]. In another report, salivary AST levels were significantly higher in a group of patients who had more severe periodontal disease than controls [48]. In addition, gingival bleeding and suppuration were observed in 20% of individuals with salivary AST concentrations three-times higher than the median of the controls[48]. Similarly, in a larger patient cohort, AST levels significantly increased with increasing severity of periodontitis, whereas ALT levels increased, but not to a significant level, above the healthy controls [35] It has also been demonstrated that salivary levels of AST and ALT in patients with periodontitis decrease significantly after scaling [49]. These findings suggest that periodontal destruction, gingival bleeding and suppuration are related to higher AST levels and possibly also to ALT levels in saliva, and confirm earlier studies that demonstrate similar increased levels of AST in crevicular fluid at sites of periodontitis [46]. Thus, salivary markers of cell injury, such as AST, appear to be useful for assessing periodontal disease.

Tissue inhibitors of metalloproteinases (TIMPs) - The activities of MMPs in body tissues, such as the periodontium, are regulated at one level by tissue inhibitors of metalloproteinases (TIMPs)[50]. This well-studied family of inhibitors consists of four members (TIMPs 1–4). TIMP-1, -2 and -4 are secreted extracellular proteins and TIMP-3 is bound to the extracellular matrix. All can inhibit MMPs. TIMPs can also exert other

functions including, but not limited to, MMP transportation and stabilization, MMP focalization to the cell surface, inhibition of angiogenesis and promotion of bone-resorbing activity[50]. The most common inhibitor, TIMP-1, is secreted by the regional cells of the periodontium (fibro-blasts, keratinocytes and endothelial cells) and by the migratory cells of the inflammatory infiltrate (monocytes/macrophages)[50]. Under natural conditions, inhibitors of MMPs are required for the normal physiological remodeling of connective tissue. The imbalance between MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs) is considered to trigger the degradation of extracellular matrix, basement membrane, and alveolar bone, and thus to initiate periodontal disease[50]. An increase in MMP and a decrease in TIMP levels initiate collagen degradation from connective tissue and alveolar bone. Lower levels of TIMPs have been found in the saliva of patients with chronic periodontitis[50] . Doxycycline treatment, combined with conventional periodontal treatment, increases salivary TIMP-1 concentration in these patients [50]

Bone remodeling biomarkers

Osteoprotegerin (OPG)/ nuclear factor kappa B (RANKL) - The osteoprotegerin is a member of the family of the tumor necrosis factor(TNF) receptor, which is expressed by osteoblasts[51]. The function of OPG is to reduce the interaction between the ligand receptor activator of nuclear factor kappa B (RANKL) and receptor activator of nuclear factor kappa B (RANK) on the cell surface. It competes with RANKL for binding to RANK,, competitively inhibiting osteoclast differentiation and activity[51]. The balance between receptor activator of NF-κB ligand (RANKL) and osteoprotegerin is critical to bone remodeling [52]. Changes in this balance are observed in periodontal disease [52]. In this context, salivary RANKL levels are significantly higher in untreated non-smoking, periodontitis patients than those who received maintenance therapy[52].

Alkaline phosphatase Alkaline phosphatase (ALP) is a nonspecific hydrolase enzyme present in all bodily tissues, but is particularly abundant in the liver, kidney and bone[53]. It is associated with the calcification process, and elevated ALP levels are commensurate with active bone remodeling[53]. Activity levels of ALP were significantly higher in pregnant women with periodontitis than those with gingivitis or a healthy periodontium [53]. In another study, it was observed that salivary ALP activity was also sig-

nificantly higher (five times) in saliva from patients with periodontal disease than controls [54].

Aspects that may affect salivary biomarkers concentration

Salivary diagnostics face many challenges before the entry into mainstream clinical care is achieved. Proteins, inhibitors and enzymes, known to be present in saliva, may obscure or destroy antigenic determinants needed for immunoassays, and many inflammatory markers and proteases are at elevated levels in patients who experience gingivitis and periodontitis

Moreover, when investigating periodontitis risks smoking status and obesity must be taken into account [55]

Smoking is a strong risk factor for periodontal disease in adults[56]. Tobacco smoking affects the immune system and impairs the host defenses by inhibiting granulocyte function[56] Smoking seems to also affect the salivary inflammatory biomarkers levels. For example Mantyla et al. reported that the mean MMP-8 concentrations in adults who smoke were lower than in non-smokers[57]. Obesity is known to affect host immunity[58]. Increased abdominal adiposity is a key factor. It has been reported that obese, hypertensive rats are more likely to have periodontitis than normal rats; the periodontal vessels of those rats show intimal thickening, indicating diminished blood flow[58]. Plasminogen activator inhibitor-1 (PAI-1), whose gene expression is enhanced in visceral fat, induces agglutionation of blood and raises th risk of ischemic vascular disease[58]. Therefore, PAI-1 may also decrease blood flow in the periodontum of obese subjects to promote periodontitis, PAI-1 system plays an important role in gingival inflammation[58].

The orthodontic treatment must also be taken into consideration. It is a treatment based on the controlled movement of the teeth by applying mechanical forces[59]. It occurs through the development of areas of stress and strain in the periodontal ligament, resulting in areas of apposition and bone resorption. This (controlled) inflammatory mechanism is dependent on the specific release of biochemical mediators associated with chronic inflammation and osteoclast stimulation, such as IL-1β and TNFα [59.60] In

vitro studies showed that during movement of the tooth there is a sharp increase in the concentrations of thse molecules.Furthermore, some authors also found significant changes in biochemical composition of crevicular fluid in vivo[6162]. Systemic diseases can also influence molecules appearing in saliva. Infectious disease states, such as HIV infection, are known to be associated with altered salivary MMP and TIMP levels [63] and serum MIP-1α levels are elevated in multiple myeloma and other lytic bone disorders[63] potentially confounding its utility in salivary diagnosis of periodontal disease Cyclic changes in salivary ALP levels have been associated with the menstrual cycle and oral lesions [64], and periodontal therapy has resulted in altered levels of inflammatory cytokines and TIMPs in saliva[65]. Additional confounders include low flow saliva rates due to dehydration, drug administration or systemic diseases that can affect/limit saliva collection, and day-to-day variations[65]. All these factors must be rigorously considered before saliva gains real-world application.

Discussion

The traditional diagnostic parameters for periodontitis are implemented owing to their ease of use, relative noninvasiveness and reliability. However, there are several limitations to this diagnostic approach. It is needed a high trained clinician with assistance to record the findings. The collection of the diagnostic information includes the use of expensive radiographic equipment that makes the procedure time and labor-intensive as well as costly to the consumer. These diagnostic parameters are excellent at determining a past history of disease but provide limited ability to determine the ongoing disease unless standardized longitudinal measures are obtained. A significant amount of damage must occur before these diagnostic parameters are able to assess the amount or severity of disease. Another limitation is the interexaminers differences when measuring parameters, due to the subjectivity of some of the analysis.In addition, there are some factors that may influence the precision of periodontal probing which are related to design and handling facility of the instrument and health of the gingival tissues, as well as experience of the clinician. Thus, periodontal probing is an imprecise technique with several potential sources of error. Furthermore, it has been clearly shown that extrinsic factors, such as smoking, can reduce the bleeding response to the periodontal probing [66]. As well, the fact that BOP could appear either from a deep periodontal tissue inflammation or to a superficial one could have important implications that should not be underestimated. Inherent difficulties in accurately assessing attachment levels include inflammation, which causes coronal displacement of the gingival margin without a concomitant migration of the dento-gingival epithelium to a level apical to the cement enamel junction, and recessions, in which an obvious loss of attachment has occurred, but there is no increase in probing depth[20].

Oral fluids have been investigated as an alternative diagnostic approach. Initially, the focus was on the oral fluids emanating from individual teeth[15]. This fluid, known as GCF, is an inflammatory exudate collected by dentists on filter paper strips. Its use has several diagnostic advantages as contributing inflammatory mediators and tissue-destructive molecules associated with periodontitis appear, and can be detected, in GCF. However, GCF analyses are time consuming by requiring multiple sampling of individual tooth sites onto filter paper strips. The procedure is labor intensive and somewhat technically demanding, requiring equipment for calibrating and measuring fluid vol-

umes. Finally, the assessment of analytes is expensive since each sample must be evaluated individually and the required assays are laboratory based and generally cannot be done chairside. In addition, GCF analyses involve miniscule amounts of fluid, often approximately 1 µl, which has an impact on laboratory analysis, and can be contaminated with blood, saliva or plaque.

The advantage of using saliva as a study specimen is that it can be easily and non-invasively collected and the collection does not require specially trained dental nurses or assistants. However, one important disadvantage of using saliva for periodontal disease diagnosis is that it fails to detect the exact site of active disease. Furthermore, variations in salivary flow rate, use of antimicrobial medications, orthodontic treatment, smoking habits or obesity can have an impact on salivary analysis. Indeed, one prerequisite for a good periodontal disease marker is its function at population level, for example independently of smoking habits or of other modifiers. Knowledge of levels of specific salivary biomarkers can, in turn, provide patients and healthcare practitioners with the ability to determine whether a disease is present, whether initiation of treatment is needed or if treatment has been successful.

It was demonstrated that the concentration of IL-1β[12 26 28 29 30], IL-6[30] and TNF-α [29 30 36], MMP-8 [44 50], AST [35 54] OPG [12 40] and alkaline phosphatase [53 54] were elevated in individuals with periodontal disease, compared to healthy controls, ALT [35 54] levels were elevated but less well characterized than AST, TIMPs [50] were decreased, MIP1α [29] levels were elevated in aggressive periodontitis, while RANKL [3640] levels were indeterminate (this may be due to the difficulty to detect soluble RANKL in saliva, because it may be bound to OPG or degraded by salivary proteases). [12 36 40]

It is generally considered that the combined use of biomarkers increases the sensitivity and specificity for obtaining accurate diagnostic information. An example of this comes from studies where diagnostic thresholds were established using elevated salivary levels of MMP-8 and IL-1β. Individually, these biomarkers are significantly associated with increased risk for periodontal disease[36]. However, their use in conjunction (diagnostic panels) demonstrates that the risk for periodontal disease is much greater when elevated salivary levels of MMP-8 and IL-1β are greater than two standard deviations above the mean of healthy control values[36].

However, an important point is that gingival inflammation itself can affect IL-1β secretion and consequently, its concentration in saliva, regardless of the individual subject's pocket depth[5].

Having an agreement on MMP-8 as a biomarker of periodontitis is limited mainly due to differences in applied methods[41]. Detection of both latent (precursor) and active forms of MMP-8 (both free and complex) in oral fluids may not necessarily correlate with periodontal disease progression, as the active form is associated with periodontitis, while the latent form is associated with gingivitis[41 67]. A time-resolved immunofluorometric assay (IFMA) has been introduced to detect active MMP-8 [41]. The IFMA method differs from the other immunodetection methods by its unique antibody, which detects both PMN- and fibroblast type MMP-8 isotypes, and especially their active forms [41].

The field of salivary diagnostics is rather new, but a growing number of reports have been published on the topic. Its emerging status is evident, in that many analytes have been investigated by a limited number of scientists; many only in cross-sectional study designs. Accordingly, a few promising analytes have been identified[11 41]. Before salivary diagnostics becomes established in clinical practice, biomarker discovery needs greater development and validation, especially with respect to which salivary biomarkers best correlate with periodontal disease.

Targeted approaches that identify key biomarkers linked to distinct biological phases of disease are needed to generate the panels required to provide the sensitivity and specificity needed for accurate and reproducible disease diagnosis. Identifying key molecules that appear in saliva during the processes of inflammation, connective tissue destruction and bone remodeling are critical to the field of salivary diagnosis and perio-dontal disease

Conclusion

Salivary diagnostic is an emerging field, that reveals a high clinical potential. Since levels of salivary analytes have the potential to reflect current disease activity and severity, salivary biomarkers derived from serum, gingival crevicular fluid and mucosal transudate can be useful for planning further treatment or/and evaluating if the current treatment is being effective.

Relevant clinical markers associated with specific biological phases of periodontal disease markers of inflammation, connective tissue destruction and bone remodeling associated with periodontitis appear in saliva. Specificity demands will likely require use of biomolecules from all three biological phases to `rule in' periodontitis and exclude other inflammatory diseases of the oral cavity. Smoking and obesity may affect the usefulness of salivary biomarker analyses and must always be considered, an eventual orthodontic treatment may further complicate the diagnosis reliability. Other factors such as systemic diseases, salivary flow rate, use of antimicrobial medications may also influence on results.

In the near future, biomarker panels are likely to gain the specificity needed for the utility of saliva as a true diagnostic fluid. However, this will only be achieved once the proper combination of markers are validated in longitudinal studies and their reliability confirmed with respect to cyclical day-to-day variations and potential confounders

Furthermore studies are needed that sample patients at regular intervals in order to capture analyte profiles seen during different phases of active disease.

This approach will hopefully allow more individualized treatment to be provided before significant tissue destruction occurs.

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