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Review

Diagnostic potential and future directions of biomarkers in gingival crevicular fluid and saliva of periodontal diseases: Review of the current evidence

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ABSTRACT

Objectives: The holy grail of biomarker research in periodontology is to develop a high impact diagnostics which have a significant impact on clinical decision-making, patient outcomes and healthcare providers. In the field of periodontal diagnostics, oral fluid-based biomarkers have been studied mainly in the gingival crevicular fluid (GCF) and saliva.

Methods: A literature search was performed using the Cochrane library and PubMed databases from 2000 to January 2017.

Results: Currently, there are more than 90 different components in the GCF that have been investigated as diagnostic and prognostic markers of periodontal disease progression involving; inflammatory mediators, markers of oxidative stress, host-derived enzymes, tissue-breakdown products and mediators of bone homeostasis. Furthermore, various biomarkers in saliva have been proposed which reveal a promising outlook for saliva as a key diagnostic medium for periodontal disease. Recent systematic reviews with high value of evidence have shown that potential salivary biomarkers can provide important complimentary diagnostic information and can be used as tests for screening diagnosis, prognosis and predicting periodontal disease progression.

Conclusion: Future developments in proteomic analysis and personalized medicine will pave the way allowing novel diagnostic tools. Still, the application into the field of dentistry will depend on how practitioners will apply this into their daily clinical practice.

Clinical relevance: Still, the application into the field of dentistry will depend on how practitioners will apply this into their daily clinical practice.

1. Introduction

Periodontal diseases are inflammatory in origin in which microbial factors induce a series of host responses that mediate inflammatory events. The inflammatory process that occurs in the periodontal tissues is considered a physiologic mechanism rather than pathology by which the host defends itself against microbial challenge through a well-orchestrated network of cells, mediators and tissues. The immune inflammatory response in periodontitis is complex and involves both innate and acquired immunity. In susceptible individuals, dysregulation of these inflammatory and immune pathways causes chronic inflammation and periodontal tissue destruction. Therefore, susceptibility to chronic inflammatory disease as periodontitis may be attributed to the uncontrolled resolution of inflammation. Since failure to return to homeostasis leads to the development of the disease, it is essential to try to fully understand the molecular and cellular events in this complex system (Cekici, Kantarci, Hasturk, & Van Dyke, 2014; Nicu & Loss, 2016).

Definitely, the hallmark of the specialty of Periodontics is applying state-of-the-art science to the diagnosis and treatment of periodontal diseases (Armitage, 2013). Periodontal disease is time consuming and expensive to treat, hence prevention, early detection and management yield considerable health-care benefit. The application of scientific evidence and patient-specific information is now considered to be central to effective clinical management of periodontitis (Kwok, Caton, Polson, & Hunter, 2012). Lack of evidence-based knowledge regarding the disease progression may lead to unintentional clinical mismanagement. Therefore, the goal of periodontal diagnostic procedures is to provide useful information to both dentists and patients regarding the present periodontal disease's type and severity which serves as a basis for treatment planning and disease monitoring during periodontal maintenance (Slots, 2013).

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Monitoring disease progression is a highly skilled and technically demanding process, involving measurement of bleeding on probing, probing depth and attachment loss coupled with radiographic assessment and visual observations. The presence of bleeding upon probing is a measure that is attached to inflammation and still is the best negative predictor of periodontal disease activity, where its absence predicts lack of periodontal tissue destruction, yet it has a low sensitivity value. Meanwhile, subjective diagnostic approaches as probing depth and attachment loss do not reflect current disease activity but only assess the past tissue destruction (Buduneli & Kinane, 2011). Accordingly, it would be highly desirable to develop reliable, innovative, simple and non-invasive diagnostic methods for early detection of active disease status and for monitoring the response to periodontal therapy (Giannobile et al., 2009).

A paradigm shift has occurred in clinical and basic scientific researches which are currently designed to improve diagnostic processes via host-based tests based on understanding the progression and pathophysiology of periodontal disease. In periodontal diagnostics, concepts have evolved in order to keep pace with advances in microbiology, biochemistry, immunology, molecular biology, genetics and connective tissue biology (Armitage, 2013). Since early detection of disease plays a crucial role in successful therapy, thus, researchers are devoted to searching for diagnostic biomarkers with high sensitivity and specificity whereby periodontal risk can be identified before extensive clinical damage has occurred (Loos & Tjoa, 2005).

It is essential for the diagnostic/screening test to have both high specificity and sensitivity. The sensitivity of a test defines its ability to appropriately identify patients with the disease. It is essential for the test to be highly sensitive e.g., a clinical test with 75% sensitivity means that it can recognize 75% of patients with the disease (true positives) but 25% with the disease might be undetected (false negatives). While specificity of a test describes the ability to properly identify patients who do not have the disease, e.g., a clinical test with 75% specificity means that 75% of patients without the disease are recognized as negative (true negatives) but 25% of patients without the disease are falsely recognized as positive (false positives) (Rathnayake, Gieselmann, Heikkinen, Tervahartiala, & Sorsa, 2017).

Biomarkers were defined as “cellular, biochemical, molecular, or genetic alterations by which a normal, abnormal, or simply biologic process can be recognized or monitored” by the biomarkers definitions working Group (2001). Biomarkers indicate health, disease, and/or response to therapy and must also be robust and proven valid in clinical studies. One of the main challenges in the field of periodontology is to discover an ideal periodontal diagnostic/prognostic biomarker which should be able to identify current disease activity, to differentiate active sites from inactive ones, to predict further disease progression and lastly to monitor the response to periodontal therapy (Buduneli & Kinane, 2011; Slots, 2013).

The holy grail of biomarker research in periodontology is to develop a “high impact diagnostics” which have a significant impact on clinical decision making, patient outcomes and healthcare providers. Potential biomarkers of periodontal disease activity would either be involved in the disease pathogenesis or released as a consequence of tissue damage during disease progression (Taylor, 2014).

The biological media for detecting periodontal disease biomarkers included; gingival crevicular fluid (GCF), saliva, serum, subgingival plaque and tissue biopsies. In the field of periodontal diagnostics, several reviews in the past two decades have analyzed biomarkers in the GCF (Armitage, 2004; Loos & Tjoa, 2005; Barros, Williams, Offenbacher, & Morelli, 2016 Wassall & Preshaw, 2016) and the saliva (Zhang, Henson, Camargo, & Wong, 2009; Kinney et al., 2011; Korte & Kinney, 2016; Jaedicke, Preshaw, & Taylor, 2016). They are particularly promising due to their ease of collection and consist of both locally synthesized and systemically derived molecules.

Based on the above mentioned data, the aim of the current study was to evaluate evidence from the current literature and highlight the future directions regarding diagnostic potential of biomarkers in GCF and saliva of periodontal disease.

2. Search strategy

A literature search was performed using the Cochrane central and PubMed database from 2000 to 19 January 2017, with the following search strategy: (“gingivitis” OR “periodontitis” OR “periodontal disease”) AND (“biomarkers” OR “markers”) AND (“‘saliva”’ OR “salivary” OR “gingival crevicular fluid”). The search was limited to the English language.

3. GCF as a source of biomarkers for periodontitis

3.1. GCF composition

GCF is a physiological fluid and an inflammatory exudate that has been recognized for over 100 years, which provides a unique window for analysis of periodontal condition. It originates from the blood vessels in the gingival connective tissue, subjacent to the epithelial lining of the dentogingival space having permeated through the diseased soft tissue of the periodontal pocket (Griffiths, 2003). The composition of the GCF is a complex combination of molecules coming from the blood, host tissues and subgingival biofilm, including; leucocytes, proteins, enzymes, tissue breakdown products, inflammatory mediators and cytokines produced locally in response to bacterial biofilm (Armitage, 2004). Consequently, GCF is considered the most promising source of biochemical disease indicators as it offers great potential reflecting the ongoing response generated by cells and tissues in the periodontium (Barros et al., 2016).

3.2. Methods of GCF collection

The methods of GCF collection may be generally divided into intracrevicular and extracrevicular approaches. In the former technique the strip is being inserted into the gingival crevice, while in the latter one the strips are paced on the gingival crevice region to decrease trauma. The intracrevicular method is more often used and could be subdivided whether the strip is inserted merely at the entrance of the gingival crevice or periodontal pocket or whether it is inserted to the base of the pocket until minimum resistance is felt. Griffiths (2003) reviewed several techniques employed for the collection of GCF. He also mentioned that the technique chosen will depend upon the aim of the study since each technique has its own advantages and disadvantages. Accordingly, the techniques were divided into three basic strategies:

3.2.1. Gingival washing methods

In this technique the gingival crevice is perfused with a fixed volume of an isotonic solution, such as Hanks’ balanced salt solution. The fluid collected represents a dilution of crevicular fluid containing both cells and soluble plasma proteins. The washing technique is particularly valuable for harvesting cells from the gingival crevice region. The main disadvantage of this technique is that not all of the fluid may be recovered during the procedure. Thus, it is impossible to accurately measure the GCF volume and composition since one cannot determine the precise dilution factor.

3.2.2. Capillary tubing or micropipettes

After the isolation and drying of a site, capillary tubes of known internal diameter are inserted into the entrance of the gingival crevice. GCF from the crevice migrates into the tube by capillary action. Since the internal diameter is known the volume of the collected fluid can be accurately determined by measuring the distance which the GCF has migrated. This technique seems to be ideal as it provides an undiluted sample of ‘native’ GCF. However, to be able to collect a reasonable

volume of fluid this might require that collection times from an individual site may exceed 30 min. Another complication of this technique is the difficulty of removing the complete sample from the tubing. This occurs either by being forced out with a jet of air or more usually by centrifugation of the tube.

3.2.3. Absorbent filter paper strips

A Periopaper strip is placed at the entrance of the crevice and the fluid migrates up the strip by capillary action. The advantages of this technique are being quick, easy to use, can be applied to individual sites and is the least traumatic when used in a correct way.

3.3. Reporting GCF volume and biomarker data

The GCF volume absorbed by Periopaper strips can be quantified using a Periotron device. The Periotron is an electronic series of instruments used by research centers throughout the world including; Periotron 600, then Periotron 6000 and currently the Periotron 8000. The Periopaper should be rapidly transferred to the Periotron immediately after the GCF sample is collected, to reduce sample evaporation. The Periotron device must be calibrated with known volumes of fluid pipetted onto Periopaper strips to generate a standard curve. The 8000 series is supplied with software for calculating GCF volumes using a 4th order polynomial regression (Wassall & Preshaw, 2016). Most researchers considered that the Periotron is most accurate within the range of approximately 0.1–1.2 µl (Griffiths, 2003).

GCF volumes usually increase with increasing inflammation in the periodontium, thus recording GCF volume is useful as a general indicator of inflammation. Nevertheless, reporting GCF volume is also useful for calculating biomarker's levels of concentration in the GCF sample. Hence, authors nowadays tend to sample GCF for a fixed period (usually 30 s) and report either total biomarker content in a 30-s sample (e.g. picogram per 30-s sample) or the concentration as calculated from the assay (e.g. picogram/ml per 30-s sample). However, it is important to note that very low GCF volumes can have a dramatic effect on the concentrations of GCF biomarker calculated. This would have a major impact especially in studies that are evaluating the GCF cytokine levels before and after the effect of periodontal therapy. In such cases, the GCF volume on the periopaper may be very low or even undetected by the Periotron devise. Consequently, it is recommended to measure GCF volume with a Periotron and then calculate both the biomarker concentration according to the original GCF volume present, as well as the total biomarker amount per 30-s sample in the GCF sample, so that a full picture is presented to the reader. Moreover, calibration studies are necessary to optimize the collection and processing of GCF samples to ensure optimal and accurate analysis. The precise methods used should be fully reported in details, so that the methodology could be reproducible by other researchers (Wassall & Preshaw, 2016).

3.4. Periodontal biologic markers in the gingival crevicular fluid

GCF is a simple noninvasive approach to access the periodontium that currently plays a significant role in periodontal research. Analysis of GCF has extremely improved our understanding of periodontal pathogenesis and healing outcomes following treatment which plays a significant role in periodontal research in the years to come. The major attraction of GCF as a source of biologic markers is the site-specific nature of the sample, containing a vast array of host-derived molecules which represent relevant risk indicators of disease activity (Wassall & Preshaw, 2016).

While searching for potential periodontal diagnostic biomarkers, GCF has been extensively explored during the last twenty years, from initially just confirming health and disease states to more recently investigating it as a potential prognostic tool (Barros et al., 2016). Based on a recent report, the signature of GCF biomarkers together with periodontal pathogens and clinical measures might provide a sensitive approach for determining periodontal disease progression. This may facilitate screening of periodontitis patients in epidemiological studies and allow estimation of periodontitis activity (Zhang, Chen, Zhu, & Yan, 2016).

Up till now, there are more than 90 different components in the GCF that have been investigated as diagnostic and prognostic markers of periodontal disease progression (Loos & Tjoa, 2005). As reviewed by Chapple (2009), host-derived biomarkers in GCF including; alkaline phosphatase, beta-glucoronidase, and cathepsin B demonstrated > 77% of diagnostic accuracy in predicting future periodontal disease activity. Moreover, MMPs-8 and −9, neutrophil elastase and dipeptidyl peptidases were correlated with the identification and activity of periodontal disease (Loos & Tjoa, 2005; Chapple, 2009). Nevertheless, established evidence from the current literature highlights that specific and sensitive biomarkers are still required for consistent diagnosis, prognosis, and clinical monitoring of periodontal tissue destruction (Buduneli & Kinane, 2011). From these various components, GCF biomarkers discussed in this review include; inflammatory mediators, markers of oxidative stress, host-derived enzymes, tissue-breakdown products, mediators of bone homeostasis and growth factors.

I. Inflammatory mediators:

a) Cytokines and chemokines:

Host susceptibility is a crucial factor in periodontal diseases pathogenesis, thus assessment of the inflammatory mediators in GCF is important for identifying patients at risk of disease activity (Barros et al., 2016). Numerous studies suggested that interleukin-1beta (IL-1β), IL-2, IL-6, IL-8, IL-17 and tumor necrosis factor-alpha (TNF-α) in GCF are reliable inflammatory biomarkers in patients with different periodontal diseases (Teles et al., 2010; Rescala et al., 2010 Rescala, Teles, Fischer, Haffajee, & Socransky, 2010; Becerik, Ozturk, Atmaca, Atilla, & Emingil, 2012; Shaker & Ghallab, 2012) and decreased markedly after scaling and root planing (Cifcibasi et al., 2015; de Lima Oliveira et al., 2012). Results from these studies might indicate a possible role for these mediators regarding periodontal tissue destruction.

One of the most studied biomarkers in the GCF is IL-1β, it is a potent bone-resorbing cytokine formerly known as the osteoclast-activating factor. Previous reports demonstrated that GCF IL-1β was elevated in active sites of periodontal disease and declined after periodontal therapy and thus can be used as a laboratory tool for assessing the activity of periodontal disease (Toker, Poyraz, & Eren, 2008; Oh et al., 2015 Oh, Hirano, Takai, & Ogata, 2015). In support with these reports, Nazar Majeed, Philip, Alabsi, Pushparajan, and Swaminathan (2016) concluded in their systematic review that IL-1β can be considered one of the most common biomarkers that give precise results which could be utilized as an indicator of periodontal disease progression.

Monocyte chemoattractant protein-1 (MCP-1) is one of the most important chemokines that causes recruitment of inflammatory cells and are thus involved in periodontal destruction. Previous investigations showed that MCP-1 and MCP-4 in GCF and saliva increased progressively with the progression of periodontal disease and decreased after treatment, hence can be proposed as potential biomarkers of disease severity (Gupta et al., 2013 Gupta, Chaturvedi, & Jain, 2013; Kumari, Pradeep, Priyanka, Kalra, & Naik, 2014). Pentraxin-3 is another inflammatory mediator involved in acute-phase reaction, which has been proposed as a 'marker of inflammatory activity in periodontal disease' in the GCF (Kathariya et al., 2013; Pradeep, Kathariya, Raghavendra, & Sharma, 2011).

In a recent systematic review with meta-analysis, Stadler et al. (2016) compared GCF cytokines/chemokines levels between healthy subjects and patients with chronic periodontitis, before and after non-surgical periodontal therapy. Evidence for significant differences between periodontal health and disease was observed for pro-inflammatory mediators including IL-1β, IL-4, IL-6, IL-17, interferon-gamma and MCP-1. Nevertheless, the authors concluded that properly powered longitudinal studies are warranted for further understanding of these biomarkers predictive value concerning increased risk of

disease progression.

b) Adipokines:

To date, a growing number of adipokines have been evaluated as periodontal disease-specific biomarkers including; visfatin, leptin, adiponectin and resistin. Previous studies reported that visfatin GCF concentrations increased proportionally with the disease severity and significantly decreased after non-surgical periodontal therapy (Raghavendra et al., 2012; Wu, Chen, Wei, Luo, & Yan, 2015). Further reports demonstrated a negative correlation between GCF leptin concentration and periodontal disease progression suggesting a protective role regarding periodontal health (Karthikeyan & Pradeep, 2007; Selvarajan, Perumalsamy, Emmadi, Thiagarajan, & Namasivayam, 2015).Most recently, Akram et al. (2017) concluded in their systematic review that resistin modulates inflammation and may be used as a surrogate measure to identify subjects at risk for chronic periodontitis. Consistent findings were previously reported showing that the increased level of resistin in the GCF can be regarded as potential inflammatory marker for periodontitis (Gokhale et al., 2014).

Other recent adipokines have been investigated in the GCF as progranulin (Priyanka et al., 2013), vaspin (Doğan, Ongoz Dede, Balli, & Sertoglu, 2016a) and chemerin (Doğan, Balli, Dede, Sertoglu, & Tazegul, 2016b) which were also considered as novel diagnostic and prognostic biomarkers for periodontal disease.

II. Host-derived enzymes:

Matrix metalloproteinases (MMPs) and tissue inhibitor of matrix metalloproteinases (TIMPs) are a family of proteinases involved in collagen degradation during periodontal tissue destruction (Sorsa et al., 2016). MMP-8 levels in GCF have been under investigation by various researchers. The analysis of MMP-8 in the GCF has proven to be a sensitive and specific unbiased biomarker for rapid chair-side that aids in early detection of periodontitis and may provide a useful tool in monitoring periodontal disease progression (Romero et al., 2013 Romero, Mastromatteo-Alberga, Escalona, & Correnti, 2013; Leppilahti et al., 2014; Sorsa et al., 2016). Other MMPs have also been investigated including MMP-3, MMP-13, and TIMP-1. GCF levels of these biomarkers significantly increased in periodontally active sites and thus were considered to have a role in diagnosing disease severity (Hernandez, Martinez, Tejerina, Valenzuela, & Gamonal, 2007; Pawar & Mehta, 2015).

In a longitudinal cohort study over a 12-month period, Kinney et al. (2014) assessed a panel of GCF biomarkers including MMP-8, MMP-9, osteoprotegerin (OPG) and IL-1β and reported significantly elevated levels with high sensitivity in patients showing periodontal disease progression. Recently, Baeza et al. (2016) also observed high diagnostic accuracies for ProMMP-2, ProMMP-9, and MMP-8 in chronic periodontitis.

Further host-derived enzymes investigated in the GCF comprise; alkaline phosphatase (Kunjappu, Mathew, Hegde, Kashyap, & Hosadurga, 2012) and myeloperoxidase (Leppilahti et al., 2014) which might also be used as biochemical markers for the detection and progression of periodontal disease. Another report suggested that increased concentrations of GCF-cathepsin K, a highly expressed cysteine protease, can be considered as a 'marker of osteoclastic activity' in periodontal disease (Garg, Pradeep, & Thorat, 2009).

III. Markers of oxidative stress:

A large body of evidence shows that oxidative stress defined by an excess of reactive oxygen species and depletion of antioxidant levels in GCF lie at the heart of periodontal tissue destruction (Chapple & Matthews, 2007). Numerous studies evaluated markers of oxidative stress in GCF of patients with chronic periodontitis (Wei, Zhang, Wang, Yang, & Chen, 2010; Esen et al., 2012; Ghallab, Hamdy, & Shaker, 2016) and observed that non-surgical periodontal therapy significantly improved the redox balance in these patients (Dede, Ozden, & Avci, 2013; Hendek et al., 2015 Hendek, Erdemir, Kisa, & Ozcan, 2015).

Lately melatonin has received considerable attention because of its antioxidant, anti-inflammatory and immune enhancing properties (Gomez-Moreno, Guardia, Ferrera, Cutando, & Reiter, 2010). Few studies showed that as the degree of periodontal disease increased, GCF melatonin levels decreased (Srinath, Acharya, & Thakur, 2010; Almughrabi, Marzouk, Hasanato, & Shafik, 2013). Recently, consistent findings reported that melatonin might be considered a useful biomarker for monitoring the severity of periodontal disease and that oxidative stress GCF biomarkers could also be used to differentiate between patients with chronic and aggressive periodontitis (Ghallab et al., 2016).

IV. Markers of bone homeostasis:

Pyridinoline cross-linked carboxyterminal telopeptide of type I collagen, receptor activator of nuclear factor-κB-ligand (RANK-L), OPG and osteopontin are among the most common studied biomarkers of bone homeostasis in the GCF. These are biochemical markers specific for bone resorption, thus represent a potentially valuable diagnostic aid which may be useful in differentiating gingivitis from active periodontal bone destruction (Sharma & Pradeep, 2007; Becerik, Afacan, Ozturk, Atmaca, & Emingil, 2011). The levels of RANK-L and OPG were examined by many investigators, where the ratio of RANK-L/OPG had a consistent tendency to increase from periodontal health to periodontitis and to decrease after non-surgical periodontal therapy (Bostanci et al., 2007; Gumus et al., 2013; Hassan, El-Refai, Ghallab, Kasem, & Shaker, 2015). Based on these studies, RANKL/OPG ratio showed promise as a discloser of periodontal disease activity.

V. Tissue-breakdown products:

Cell adhesion molecules are cell surface proteins involved in the binding of cells to each other, to endothelial cells, or to the extracellular matrix. Changes reported in the levels of cell adhesion molecules in patients with periodontitis may be a sensitive indicator to differentiate healthy sites from those with periodontitis. Accordingly, these soluble adhesion molecules might be useful markers for monitoring periodontal wound healing and for the identification of periodontal disease progression (Chaturvedi, Gupta, Jain, Das, & Prashar, 2015).

Moreover, calprotectin is a major cytosol protein of leukocytes which has been thought to be a marker of inflammatory disease. Previous data indicated elevated calprotectin levels in GCF of both chronic and aggressive periodontitis, suggesting that it might be a useful diagnostic biomarker for evaluating the extent of periodontal inflammation, predicting disease activity and monitoring periodontal treatment (Becerik et al., 2011).

Recently, periostin was discovered as protein highly expressed in periosteum and periodontal ligament that might have a protective role against periodontal disease. Levels of periostin in GCF and saliva may be used as a possible biomarker to evaluate the outcome following non-surgical periodontal therapy in patients with chronic periodontitis (Kumaresan, Balasundaram, Naik, & Appukuttan, 2016) may have a promising diagnostic potential for the aggressive forms of periodontal disease (Aral, Koseoglu, Saglam, Pekbagriyanik, & Savran, 2016).

VI. Growth Factors

Growth factors have also been investigated in GCF in relation to periodontal disease. It has been suggested that changes in the GCF levels of transforming growth factor-beta might be useful for monitoring the progress of periodontal repair and regeneration (Kuru, Griffiths, Petrie, & Olsen, 2004) and may as well predict the progression of periodontitis (Khalaf, Lonn, & Bengtsson, 2014). Similarly, vascular endothelial growth factor has attracted attention as a potential inducer of angiogenesis that could be considered as a biomarker of periodontal disease progression (Sakallioglu, Sakallioglu, Lutfioglu, Pamuk, & Kantarci, 2015). Furthermore, hepatocyte growth factor was proposed to play an important role in the progression of periodontitis by stimulating growth of epithelial cells and preventing regeneration of the connective tissue attachment, thus might be regarded as another biomarker for periodontal disease activity (Anil et al., 2014).

4. Saliva as a source of biomarkers for periodontitis

4.1. Saliva as a diagnostic tool

Saliva is a unique oral fluid that consists of a mixture from the major and minor salivary glands. It also includes constituents of non-salivary origin derived from GCF, expectorated bronchial secretions, serum, blood cells from oral wounds, as well as bacteria and bacterial products, viruses and fungi, desquamated epithelial cells and food debris (Nomura et al., 2012). In addition, saliva as a mirror of oral and systemic health serves as an attractive vehicle and is appealing for use as a diagnostic fluid for oral-related disease due to its many advantages over other diagnostic bodily fluids (Kim, Kim, & Camargo, 2013).

While GCF has several diagnostic advantages, nevertheless, diagnostic tests for periodontal disease based on samples from the gingival crevice have not received extensive approval by clinicians. There are many reasons for this, including; time consuming, requiring multiple sampling of sites, blood contamination and unrealistic expectations regarding the accuracy of the tests. Finally, analysis is expensive and cannot be done chair side (Kaufman & Lamster, 2000).

On the other hand, collection and analysis of salivary biomarkers offers to resolve some of the problems inherent in sampling GCF. Saliva is more easily accessible, abundant, sampled in a much larger volume without needing clinical facilities and no complex skills are required for proper sampling. Moreover, saliva has elements which reflect the activity of all periodontal sites and therefore its content reflects a consensus 'whole mouth' inflammatory status rather than at active disease sites as with GCF analysis (Buduneli & Kinane, 2011; Jaedicke et al., 2016).

Based on the above mentioned data, salivary diagnostics offer an easy, simple, rapid and safe approach for disease detection, which also makes clinical trial involvement more attractive for the patients. Accordingly, a salivary diagnostic tool can serve as a non-invasive, sensitive, specific and useful test that can aid in screening large populations and offers potential for the prediction of periodontal disease progression or stability (Zhang et al., 2009; Kinney et al., 2011).

With this objective in mind, a chair-side saliva test could play a very important role in determining which patient and what specific sites are in need of periodontal therapy so that the tissue destruction process is prevented or arrested. Although challenges remain ahead, the use of saliva-based oral fluid diagnostics appear to hold promise for future application to diagnose periodontal diseases and to predict periodontal treatment outcomes providing a high potential to revolutionize the next generation of diagnostics (Ji & Choi, 2015).

4.2. Collection of saliva

The fluid mostly collected for salivary diagnostic purpose is expectorated whole saliva, a mix composed largely of the secretions from the major salivary glands along with the modest contributions from the minor salivary glands and gingival crevicular fluid. There are two methods for saliva collection; unstimulated whole-mouth saliva (UWS) and stimulated saliva. Unstimulated or resting saliva is usually collected by passive drooling into a graduated tube or preweighed vial so that flow rate per unit time can be measured (Navazesh, 1993). The use of UWS method is preferred as a biomarker fluid as it avoids the potential difference generated by using various types and intensities of reflex stimulation. However, one of the main drawbacks of using UWS is the low volume of fluid obtained, especially in geriatric subjects and those complaining from xerostomia Proctor (2016).

4.3. Periodontal biologic markers in the saliva

Saliva contains a highly enriched content of proteins, genetic molecules and locally and systemically derived biomarkers of periodontal disease that can be analyzed. Various biomarkers in saliva have been proposed which reveal a promising outlook for saliva as a key diagnostic medium for determining periodontal disease. Numerous reviews have shown that potential salivary biomarkers can provide important complimentary diagnostic information and can be used as tests for screening diagnosis, prognosis and predicting periodontal disease progression (Kaufman & Lamster 2000; Zhang et al., 2009; Giannobile et al., 2009; Nomura et al., 2012; Jaedicke et al., 2016; Korte & Kinney, 2016;). However, few studies have longitudinally monitored salivary biomarker profiles in patients with respect to periodontal status or determined if salivary biomarkers accurately represent periodontal disease status over time (Thomas et al., 2009).

Matrix metalloprotinase-8 is regarded as one of the promising candidates for diagnosing and predicting the progression of periodontal disease in saliva (Zhang et al., 2009). Elevated levels of salivary MMP-8 have repeatedly demonstrated significant positive correlations with periodontal clinical parameters in several studies (Miller, Langub, Kryscio, & Thomas, 2006; Ramseier et al., 2009; Costa et al., 2010; Gursoy et al., 2010; Kinney et al., 2011;). Moreover, significant reductions in salivary MMP-8 levels have been found after non-surgical periodontal therapy, suggesting their potential ability to monitor periodontal disease activity (Sexton, Lin, Kryscio, Ebersole, & Miller, 2011). Furthermore, a portable diagnostic hand-held point-of care-device, measured the oral fluid MMP-8 concentrations which were significantly elevated in periodontitis patients and decreased after scaling and root planing (Herr et al., 2007).

Similar diagnostic power has also been demonstrated for pro-inflammatory cytokines which mediate osteoclastogenesis and bone breakdown, such as IL-1β, IL-6 and TNF-α. Significantly elevated levels of these markers were observed in active periodontal disease sites (Miller et al., 2006; Costa et al., 2010; Gursoy et al., 2011) and also decreased after periodontal treatment (Sexton et al., 2011), therefore, they might serve as biomarkers of periodontitis.

Advances in technologies allowed researchers to identify potential panels of combined salivary biomarkers and periodontal pathogens which are more robust in distinguishing patients with periodontitis from healthy individuals as well as predicting future disease progression and stability. Ramseier et al. (2009) observed that differences in periodontal disease severity were efficiently detected by a combination of MMP-8, −9, OPG and calprotectin assays coupled with quantification of red complex bacteria in dental plaque. Sexton et al. (2011) also examined salivary biomarkers involved in inflammation, connective tissue degradation and alveolar bone turnover and revealed that MMP-8, OPG, macrophage inflammatory protein-1 alpha, IL-1β, IL-8 and TNF-α reflected disease severity. Moreover, MMP-8 was the stand out as the best biomarker indicative of response to therapy. While Kinney et al. (2011), demonstrated that MMP-8, −9, OPG and IL-1β in low concentrations, successfully predicted periodontal stability. Another novel diagnostic approach revealed that the combinatorial ability of Porphyromonos gingivalis, IL-1β and MMP-8 altogether were able to detect periodontitis more accurately than each marker alone (Gursoy et al., 2011).

In a recent review, Jaedicke et al. (2016) concluded that IL-1β and hepatocyte growth factor are the most robust salivary biomarkers for periodontal disease studied up until now. Nevertheless, analysis of multiple salivary cytokines has shown inconsistent evidence regarding a 'biomarker signature'. Therefore, high-quality research designs targeting sensitivity and specificity are mandatory to evaluate if the salivary biomarker can be utilized as a diagnostic test for early detection of periodontitis.

On the other hand, salivary markers of oxidative stress are widely debated as a probable tool for periodontal diagnostics. Oxidative stress markers have been extensively studied and found to be biomarkers of disease activity (Baltacioglu et al., 2014; Banasova et al., 2015; Villa-Correa, Isaza-Guzman, & Tobon-Arroyave, 2015). Novakovic et al. (2014) further stated that non-surgical periodontal therapy affected total antioxidant capacity in saliva. The local involvement of melatonin

in the pathogenesis of periodontitis due to its antioxidant abilities, left it proposed as a salivary risk indicator for the severity of periodontal disease (Sirnath et al., 2010; Almughrabi et al., 2013). Salivary melatonin levels were also recovered after periodontal therapy and correlated with a decrease of local periodontal inflammation (Bertl et al., 2013).

In addition, other biomarkers as alkaline phosphatase, aspartate aminotransferase, RANKL/OPG, visfatin, chemerin and soluble CD44 (a cell surface adhesion molecule that mediates neutrophil adhesion and transendothelial migration) have been identified in the saliva and their elevated concentrations were associated with periodontal destruction (Ghallab & Shaker, 2010; Nomura et al., 2012; Tobon-Arroyave, Isaza-Guzman, Restrepo-Cadavid, Zapata-Molina, & Martinez-Pabon, 2012; Tabari, Azadmehr, Nohekhan, Naddafpour, & Ghaedi, 2014; Ozcan, Saygun, Serdar, & Kurt, 2015; Hassan et al., 2015).

5. Methods of biomarkers analysis in the GCF and saliva

Various methods have been used for detecting and quantifying biological markers in GCF and salivary samples, involving; bioassay, radioimmunoassay and enzyme-linked immunosorbent assay (ELISA). In the field of periodontal diagnostics, ELISA-based technologies are almost universally used in research for assaying fluid-based biomarkers (Jaedicke et al., 2016). ELISAs are sandwich-type immunoassays used to detect the presence of a monoclonal antibody adsorbed onto wells of microtiter plates that binds to the test antigen. The color intensity in the reaction wells is determined by optical density scanning of the plate and the quantity of the test antigen is determined by comparison with a standard curve included in each assay kit based on the manufacturer (Wassall & Preshaw, 2016).

ELISA is the most sensitive, well-established and widely available biochemical diagnostic tool used in periodontal research. ELISA-based techniques seem most cost-effective because they are more simple and inexpensive to perform. The main advantages of ELISAs include high throughput and high reproducibility, it also allows for antigen detection using extremely small samples (Jaedicke et al., 2016). Moreover, sensitivity of ELISA was reported to be superior to other techniques e.g. radioimmunoflourescence as it gives identical results to those of radioimmunoassay without the hazards of radioactive reagents (Lequin, 2005). Usually, only a single ELISA can be used for assessing a single GCF sample. Hence, two basic multiple assays have been established using the ELISA technology for simultaneous quantification of multiple cytokines including planar array assays and microbead assays. However, the high cost of these multiplex assays limit their use in large clinical studies (Wassall & Preshaw, 2016). Furthermore, evidence suggest that analytical techniques with greater sensitivity and linear range may unmask periodontitis-associated biomarkers that could not be detected by conventional ELISAs (Christodoulides et al., 2007).

6. Limitations of biomarkers for periodontal diagnosis

Given the complex nature of periodontal disease, the identification of a single sensitive and specific diagnostic marker for disease detection and prediction seems illusionary (Zhang et al., 2009). Therefore, analysis of the combination of markers may provide a more accurate assessment of the periodontal patient and could be useful in identifying biomarkers that predict progression. Unfortunately, only a handful of GCF and salivary tests have made their way into clinical practice and no clinical or laboratory test is routinely employed in the monitoring of patients with periodontal disease (Kaufman & Lamster 2000; Buduneli & Kinane, 2011).

Even after the development of highly sophisticated methods and almost 30 years of research, no adequate marker has resulted in major conceptual changes in the field of periodontal diagnostics (Armitage, 2013). Meanwhile, most of the work came short of providing clinically reliable and useful information for practitioners in terms of developing a more precise periodontal diagnosis and subsequent treatment planning (Kim et al., 2013).

In addition, most studies investigating the relationship between salivary and GCF biomarkers and periodontal diseases study designs were cross-sectional. Hence, they just reported associations of biomarkers with the presence of periodontal disease, testing whether or not a particular marker is discriminatory, which merely reflects historical disease activity rather than current processes (Jaedicke et al., 2016). Besides the presence of few longitudinal studies available in the literature measuring the value of biomarker on disease progression, another dilemma the periodontal clinicians are now facing is how to detect periodontal disease progression clinically. It is obvious from the currently available evidence that we do not have a reliable clinical way to measure disease progression, which is a major confounding for the use of biomarker for disease prognosis and assessment.

Moreover, studies in the literature have a lot of methodological limitations with a small sample size, making it difficult to draw proper conclusions. Consequently, this decreases the statistical power of the study and the probability of establishing any causal relationship between the analyzed biomarkers and periodontal disease questioning both their external and internal validity (Rathnayake et al., 2017).

7. Future directions for oral fluid biomarkers

7.1. Personalized medicine in periodontics

Personalized medicine is a medical model that uses genetic, genomic, environmental and clinical diagnostic testing to individualize patient care. A combined analysis is required to identify the set of biomarkers with the most favorable combination of sensitivity, specificity, reproducibility and correlations with established disease diagnostic criteria. Utilization of this model in oral health care, specifically in periodontology, has the potential to provide discriminating patient-stratification models to develop highly individualized diagnosis, prognosis and personalized treatment (Giannobile, 2012). Personalized medicine for periodontal diseases using saliva will be soon developed to make proper clinical decisions regarding disease susceptibility, site-specific risk of disease progression and treatment modalities (Giannobile, Kornman, & Williams, 2013). The future is bright for the use of rapid, easy-to-use diagnostics providing an enhanced patient assessment that will allow oral health-care providers to improve prevention and treatment of periodontal diseases (Miller et al., 2010; Korte & Kinney, 2016).

7.2. Point-of-care diagnostics

Point-of-care (POC) diagnostics is defined as a medical testing that is not performed in a laboratory, yet at the patient's home, or the doctor's office. Optimally, such tests should be available in the form of chair-side or home-use dip-stick tests. By a POC device using saliva patients could easily diagnose periodontitis at home and visit their periodontist accordingly (Ji & Choi, 2015). These self-performed tests should accelerate clinical decision-making and monitoring of periodontal disease progression (Kaufman & Lamster, 2000).

In the field of periodontal diagnostics, recent developments in POC testing with new technologies have advanced significantly for the future use of oral fluids allowing accurate, rapid chair-side testing and enhance individualized care. Hereafter, researchers have been searching for explicit markers of periodontitis in saliva and GCF for the development of adjunctive, non-invasive, novel technologies (Christodoulides et al., 2007; Miller et al., 2010; Giannobile, 2012). Currently, the activity MMP-8 lateral-flow POC immunotests is a recently developed commercially available mouth-rinse that is practical, convenient and inexpensive test that takes just 5 min that is used to detect, predict and monitor the course and treatment of periodontitis (Heikkinen et al., 2016; Rathnayake et al., 2017). This test is one of the

few tests, that qualifies for a biomarker which could identify disease activity, predict progression and monitor response to periodontal therapy.

The focus has been on the development of miniature-sized 'chemical processing units' that process fluids and provide information that is relevant to the inflammatory, connective tissue-degradation and bone-loss phases of periodontitis, such as lab-on-a-chip, microarray and microfluidic devices for screening and risk assessment. Lab-on-a-chip methods incorporate sampling, sample preparation, detection and data analysis all in one small device. While microfluidics-based devices can analyze oral fluid samples like GCF and saliva. Diagnostic targets detected by POC technologies include nucleic acids, proteins, metabolites and other small molecules. Nevertheless, one of the greatest challenges is not from bench to chair-side, but from chair-side to clinical practice (Sexton et al., 2011; Sackmann, Fulton, & Beebe, 2014).

7.3. Proteome analysis

Proteomics is a powerful approach in biomedical research because it directly studies the key functional components of biochemical systems, namely proteins. Wilkins et al. (1996) introduced the word "proteome", which was an amalgamation of two words which are "protein" and "genome". The proteome is the protein complement of the genome, and proteomics is analysis of the portion of the genome that is expressed. The value of biomarkers has been recognized and extensively explored using proteomic methods. These approaches are anticipated to be a useful biochip array amenable to low-cost POC devices (Wignarajah et al., 2015; Kuboniwa et al., 2016).

Advances in proteomics technology based on analysis of GCF proteome might have conclusive prognostic and diagnostic value leading to the identification of novel biomarkers of periodontal health or disease. Currently, there are limited data available in the literature regarding proteomic analysis of GCF. The proteomic analysis of GCF in different periodontal conditions demonstrates marked differences according to disease profile. Zelko, Mariani and Folz (2002) found a total of 327 GCF proteins in periodontally healthy individuals using a gel-free method that were analyzed directly by liquid chromatography–tandem, suggesting that they may be used as a reference in future proteomic studies on GCF biomarkers of periodontal disease. Later studies reported that up to 432 different proteins have been identified in GCF samples (Baliban et al., 2012; Silva-Boghossian et al., 2013). Considering that the protein composition of GCF might reflect the pathophysiology of periodontal disease progression, GCF protein profiles obtained from healthy-looking individuals may be explored as standard GCF proteomic patterns, which might serve as a reference for the identification of periodontal diseases biomarkers by proteomic analyses. However, further studies with larger sample sizes are needed to validate the role of the identified proteins in the pathogenesis of periodontal disease (Barros et al., 2016).

Searching for markers to predict health or disease, Bostanci et al. (2010) used quantitative proteomic analysis with liquid chromatography–mass spectrometry to analyze GCF samples and reported that GCF proteins cystatin-B and alpha defensin 1 were detected only in healthy samples, while L-plastin, a protein with a vital role in immune-mediated events, was only detected in GCF of aggressive periodontitis patients. Furthermore, Baliban et al. (2012) used high-throughput proteomic analysis to detect biomarker combinations in GCF samples aiming to predict health or disease. Their findings indicated that advances in proteomics technology can contribute to the development of tools to predict the periodontal status of individuals based on analysis of GCF samples. Later on, Baliban et al. (2013) proposed a large-scale proteomic analysis and mixed-integer linear optimization that provided new insight into the identification of novel combinations of GCF-derived sets of biomarkers which can accurately discriminate between periodontal health or disease with greater than 95% predictive accuracy. As recommended by the authors, these proposed combinations of biomarkers should be further validated in future studies considering different periodontal conditions as well as changes related to periodontal therapy. Their observations showed that advances in proteomics technology for analysis and prediction of biomarkers could markedly aid in emerging reliable tools in the field of periodontal diagnostics. In his recent review, Barros et al. (2016) concluded that metabolomic analyses of GCF that measure microbial and host interactions associated with periodontal disease have the potential utility to expand our understanding and improve the landscape for the discovery of diagnostic, prognostic and therapeutic markers.

A key advance in this area is the development of the salivary proteome knowledge base which is part of the human salivary proteome project. Salivomics is a developing branch which describes the study of biological molecules like the transcriptome, the proteome and the metabolome in saliva which will launch the personalized diagnostic approaches in dental clinics (Wong, 2012). The main advantages of salivary proteomics are that low levels of a specific biomarker can be detected. These handheld, automated and easy-to-use systems will enable rapid detection of salivary protein biomarkers that can be used for POC disease screening and detection (Haigh et al., 2010).

Proteomics holds promise for disease-associated biomarker identification and extensive progress has been made in the proteomic analysis of saliva through a combination of sophisticated approaches to protein separation and advances in mass spectrometry technology. The salivary proteome has now been identified; it contains some 1166 proteins (Denny et al., 2008) and has the potential to open up new doors for the discovery of oral disease biomarkers for early detection and monitoring of disease status (Zhang et al., 2009). Accordingly, the use of proteomic analysis of whole saliva for the identification of periodontal diseases offer significant potential for providing individual "signatures" for risk of disease progression. The inclusion of proteomics will potentially improve the diagnosis and treatment of periodontal diseases (Salazar et al., 2013).

8. Genetics and periodontal disease

Chronic periodontitis is considered as a complex genetic disease, yet challenges exist to develop clinically relevant diagnostic tests for periodontal diseases, because genetic polymorphisms that contribute to disease susceptibility are individually not deterministic of the disease. Previous studies have offered a valuable impact on understanding the genetic basis of periodontal disease; still specific candidate genes of susceptibility are unknown. Although genome-wide studies and screening of single-nucleotide polymorphisms demonstrated new genetic information, reports have focused only on evaluating genetic polymorphisms (Taba, Souza, & Mariguela, 2012). Several genetic polymorphisms have been studied for their association with chronic periodontitis, including ILs genes, vitamin D receptor and TNF-β gene (Kinane & Hart, 2003; Vijayalakshmi, Geetha, Ramakrishnan, & Emmadi, 2010). Nevertheless, none of these studies provided strong diagnostic or prognostic markers to identify patients within the general population who are at risk of periodontal disease. In a more comprehensive study, Demmer et al. (2008) analysed the whole genome to show the differential gene expression of healthy and diseased periodontal sites and observed thousands of dysregulated genes in disease compared to health. Still, the applicability of this data in diagnostic tests is limited. It is noteworthy that understanding the relationship between genetics and periodontal disease progression has indeed provided valuable information for the identification of disease biomarkers that might have a potential diagnostic value (Giannobile, 2012; Taba et al., 2012).

9. Conclusion

Future developments in tissue engineering, gene therapy and biopharmaceuticals will pave the way allowing novel therapeutic

opportunities. However, the application into the field of dentistry will depend on how practitioners will apply this into their daily clinical practice (Gupta, Govila, & Saini, 2015). Still, much work remains to identify molecules with clinical utility for estimating current and future destructive periodontal disease activity. With the current picture, it is obvious that highly specific and sensitive biomarkers for diagnosis and monitoring of periodontal diseases are still required (Buduneli & Kinane, 2011). In a recently conducted systematic review, Nazar Majeed et al. (2016) concluded that it is quiet early to depend on oral biomarkers alone in the diagnosis of periodontal disease, particularly since there is no universal methods for their collection and analysis. However, these biomarkers can be used as an adjunctive method for the clinical parameters which are still considered the most reliable methods for diagnosis and monitoring periodontal disease progression.

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