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# Oral Fluid Biomarkers in Smoking Periodontitis Patients and Systemic Inflammation

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Additional information is available at the end of the chapter

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## 1. Introduction

Periodontitis is a chronic destructive condition caused by periodontopathogenic bacteria and inflammatory response combined with immune system effects characterized by gingival inflammation and loss of periodontal attachment and alveolar bone (Irfan et al. 2001). Host response is modified by genetic and environmental factors such as smoking (Seymour and Taylor, 2004), which has proven to be the very important risk factor for chronic periodontitis in adults (Genco and Borgnakke, 2013) as well for adolescents (Heikkinen et al. 2008, Heikkinen 2011). In periodontitis host response indeed plays an important role in the destruction of connective tissue and bone (Graves 2008). Smoking affects the immune system and impairs host response by several mechanisms both systemically and locally in saliva and gingival crevicular fluid (GCF). Systemically smoking increases the number of neutrophils in peripheral blood but their ability to migrate through capillary walls is impaired (Hind et al. 1991).

Several types of inflammatory biomarkers associating both with oral diseases and systemic diseases have been detected in saliva and GCF. These include interleukins-1 $\beta$ , -6 and -8 (IL-1 $\beta$ , -6 and -8), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and matrix metalloproteinases (MMP)-8 and -9 (Fox 1993, Kaufman and Lamster 2000, Seymour and Gemmell 2001, Kaufman and Lamster 2002, Miller et al. 2006, Rathnayake et al. 2012 and 2013), and tissue inhibitors (TIMP)-1 of metalloproteinase (Seymour & Gemmel, 2001). Several studies have shown the association between increased MMP-8 levels and chronic periodontitis (Mäntylä et al. 2006, Kraft-Neumärker et al. 2012, Leppilähti et al. 2011). Matrix metalloproteinases (MMPs) and TIMP-1 might be candidates for monitoring periodontal status in smokers and non-smokers from oral fluids, such as GCF and saliva. GCF has a particular role in site specific diagnosis. However,

saliva is easy to collect and thus more practical. Saliva is mainly composed of water (98%), and other compounds (2%) are electrolytes, glycoproteins, antibacterial compounds, and various enzymes. This unique biological fluid has multiple functions, such as rinsing, solubilisation of food substances, food and bacterial clearance, lubrication of soft tissues, bolus formation, dilution of detritus, swallowing, speech and facilitation of mastication, all of which are related to its fluid characteristics and specific components. In addition, saliva components contribute to mucosal coating, digestion and antibacterial defence (Lee & Wong, 2009).

Periodontitis is a major health problem involving 10% to 60% of population depending on definition (Albandar and Rams 2002) and it is traditionally diagnosed clinically and by radiographical examinations. New methods based on oral fluid inflammatory markers have been suggested for diagnosing oral diseases and inflammation associated with systemic diseases. However, smoking has an effect on levels of several possible diagnostic biomarker candidates. Thus the aim of this chapter is to clarify the diagnostic meaning of oral fluid inflammatory biomarkers in periodontitis in smoking adolescents and adults. We also discuss systemic inflammation and possibilities to analyze it with specific biomarkers in saliva.

## **2. Smoking as a modifier of the host defense**

Cigarette smoking is a principal modifiable environmental risk factor for periodontitis (Palmer et al. 2005). It affects the immune system by impairing host defense by inhibiting granulocyte function (Söder et al. 2002) and by neutrophil respiratory burst which causes oxidative stress in tissues (Chapple and Matthews 2007). According to previous study results by Matthews et al. (2011) cigarette smoking seems to have two-sided effect on periodontal inflammation: on one hand smoking has an effect on oxygen depletion with tissue damage and on the other hand it impairs the ability of neutrophils to response to subgingival periodontal bacteria.

Smoking decreases both the inflammatory infiltrate and number of dendritic cells (DCs) in chronic gingivitis (Souto et al., 2011). In addition it seems that smoking decreases CC chemokine ligand (CCL)3 and CXC chemokine ligand (CXCL)8, while CC chemokine ligand (CCL)5 seems to be increased in chronic periodontitis (Souto et al., 2014). Impaired neutrophil chemotaxis is observed in smokers compared to nonsmokers too (Srinivas et al. 2012). Mature DCs are involved in the production of inflammatory cytokines and Th1/Th2/Th17 immune responses in periodontal disease (Cutler and Jotwani, 2004; Allam et al., 2011). Nicotine seems to play an important role in host immune modulation. DCs differentiated in the presence of nicotine and stimulated by lipopolysaccharide induced a differentiation of naive CD4 T cells into Th2 cells. However, DCs differentiated without nicotine and stimulated by lipopolysaccharide induced Th1 immune responses (Yanagita et al. 2014).

## **3. Effects of smoking on oral inflammatory biomarkers**

Reduced neutrophil chemotaxis and impaired phagocytosis in smokers have been shown in several studies suggesting that smokers' periodontal defence is defective compared with non-

smokers (Johannsen et al. 2014). This may also be reflected in GCF and salivary content of biomarkers in smokers, which is relevant when possible point-of care diagnostic application is considered. However, the intensity and duration of smoking may also have an effect on GCF biomarker levels, but studies which take into consideration different smoking history are lacking.

In a study of Stein et al. (2006), where GCF proteins were profiled by a protein chip technology, spectral fingerprints were significantly different between smokers and non-smokers. Several spectral peaks were detected only from GCF of smokers suggesting that some proteins are there over-expressed and could potentially serve as biomarkers (Stein et al. 2006). Several studies have reported that smoking either inhibits or intensifies individual biomarkers in GCF, but contradictory findings do exist concerning some biomarkers. This underlines the effect of differences in GCF sampling and analysing methods and other study specific factors, which may lead to inter-study variation in detected biomarker levels

MMP-8, MMP-8/TIMP-1 ratio, IL-1B, myeloperoxidase (MPO), elastase, OPG and some bacterial biomarkers, so called red complex species *Tannerella forsythia*, *Porphyromonas gingivalis*, *Treponema denticola*, and *Aggregatibacter actinomycetemcomitans* have proven diagnostic properties to differentiate periodontitis from healthy sites in multiple independent studies. This has been shown both at site level in GCF samples and at patient level in saliva or mouthrinse samples (Hernandez et al. 2010, Kraft-Neumärker et al. 2012, Leppilahti et al. 2011, Leppilahti et al. 2014a,b, Mantyla et al. 2003, Nwhator et al. 2014, Ramseier et al. 2009, Rathnayake et al., 2013; Sexton et al., 2011). Analyzing of multiple biomarkers simultaneously can give even better diagnostic performance (Gursoy et al. 2011, Ramseier et al. 2009).

Nevertheless, biomarkers mentioned above have clear diagnostic properties for periodontal diseases, but many oral fluid biomarkers exhibit large variation of detected levels in both healthy and diseased sites ((Kraft-Neumärker et al 2012, Leppilahti et al. 2014, Mantyla et al. 2003, 2006). Modifying factors, such as smoking, may have an effect on the GCF biomarker levels and disturb the diagnostic interpretation, if these factors are not taken into account (Heikkinen et al. 2010, Heikkinen et al. 2012, Leppilahti et al. 2014a,b). Another reason for large variation is caused by the nature of the periodontitis itself. Progression of periodontitis is regarded to consist of quiescent periods followed by randomly occurring bursts of activity (Goodson et al. 1982, Socransky et al. 1984). Large variation of levels of inflammatory biomarkers can also be an indication of the fluctuating characteristic of periodontitis. Inflammatory GCF biomarker levels can be low in periodontitis sites during the quiet period, but levels can multiply exponentially during the burst of activity (Leppilahti et al. 2014a,b, Sorsa et al. 2010, Mäntyla et al. 2006). This means that most of GCF biomarkers do not associate with periodontitis in a linear and deterministic way, and it is the dynamics between the bursts and quiet periods that matters (Papantonopoulos et al. 2013, Papantonopoulos et al. 2014). In addition possible biomarker candidates may have a role in normal physiologic tissue regeneration. Thus, biomarkers can be used as diagnostic tool only if we can define the range of physiological levels and the cutoff for pathological bursts. One definite cutoff for a biomarker is not realistic, however, and modifying factors should be taken into account. For example, even in stable periodontitis sites after successful treatment MMP-8 levels are higher compared to healthy

controls (Mäntylä et al. 200, Sorsa et al. 2010). In addition, modifying factors, such as smoking and pregnancy, has to be taken into account (Gürsoy et al. 2008, 2010, Heikkinen et al. 2010, Leppilähti et al. 2014).

Saliva would be a non-invasive sample material for oral and periodontal diagnostics. However, it is less specific than GCF and should be regarded to give a more general picture of oral health. As an example of potential diagnostic capacity of whole saliva is a study where whole saliva periodontitis associated proteome was analysed (Salazar et al. 2013). Twenty proteins were present in different abundance levels in the periodontally healthy subjects and periodontitis patients. And further, nineteen out of these 20 proteins showed higher intensities in periodontitis saliva, and eight were previously reported potential periodontitis biomarkers, among others MMP-8. Also specific protein signatures displayed characteristics of chronic periodontitis. However, effect of smoking should also be considered when salivary or oral rinse sample biomarker levels are analysed.

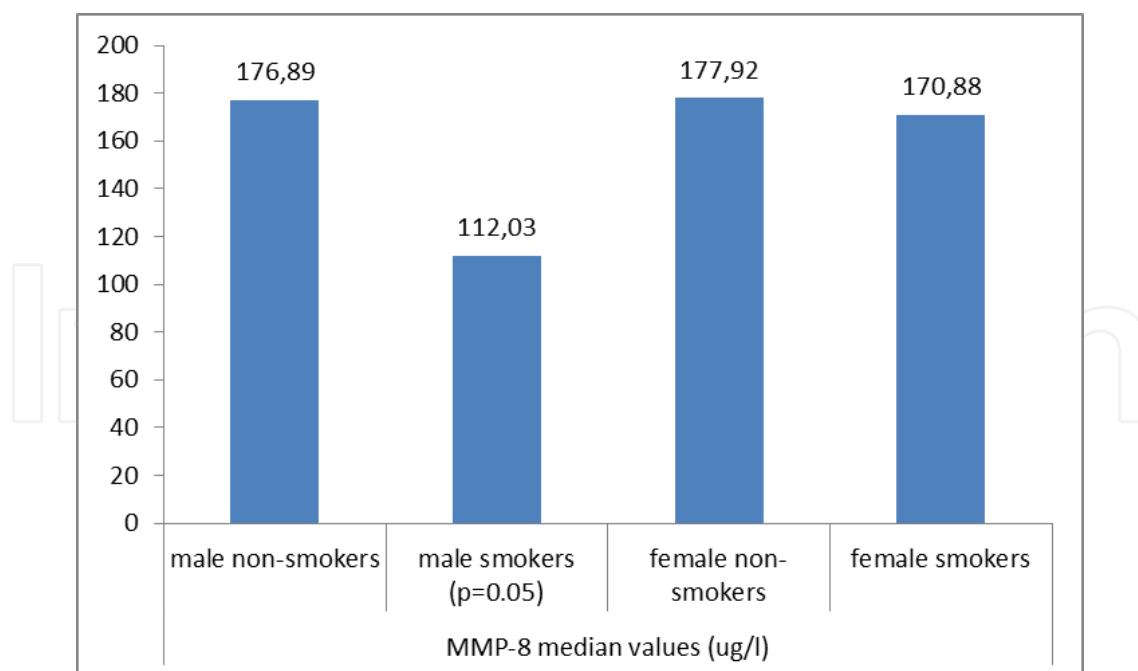
### 3.1. Matrix Metalloproteinase (MMP) -8

MMP-8 is the major collagenase in GCF, and point-of-care diagnostic tests have been developed based on analysing it (Sorsa et al. 1999; Prescher et al. 2007, Mäntylä et al. 2003, 2006, Sorsa et al. 2010). The tendency to lower MMP-8 concentrations in GCF of smokers compared to non-smokers has been observed (Persson et al. 2003) as well as lower salivary levels of MMP-8 in current smokers (Liede et al. 1999). This should be noticed when diagnostic use of MMP-8 is being considered. However, the effect of smoking on GCF MMP-8 levels seems to be two-fold. While overall MMP-8 mean level tends to be lower in GCF of smokers when compared with non-smokers, in progressing attachment loss during the maintenance phase the MMP-8 concentrations of smokers are at the same level as in non-smokers (Mäntylä et al. 2006; Leppilähti et al. 2014a). In these studies, when sites were explored in respect of repeatedly substantially elevated MMP-8 concentrations during the maintenance phase, in part of smokers' sites MMP-8 concentrations reached the highest levels of all sampled sites. Thus, lower level of MMP-8 in smokers' GCF does not relate to all sites or to all smoking periodontitis patients. For this reason when MMP-8 is considered as target for point-of-care diagnostic test different cut-off levels for MMP-8 detection should be considered for smokers and non-smokers (Leppilähti et al. 2014a). When biomarkers in saliva samples were detected and compared with periodontal health status regarding smoking as dichotomous yes-no parameter, in smokers' saliva concentration of IL-8 and MMP-8/TIMP-1 ratio were lower than in non-smokers, and salivary MMP-8 had a borderline p-value significantly lower in smokers (Rathnayake et al. 2013a). Possible explanation was considered to be the lower GCF flow of smokers, but also that the effect of smoking on periodontal inflammatory cells is reflected in saliva. In another study salivary concentration of MMP-8 differentiated periodontitis patients from controls, but in periodontitis patients who were smokers this difference was lost; however, the combination of MMP-8 and ICTP and the MMP-8/TIMP-1 ratio differentiated periodontitis cases from controls suggesting, that a combination of biomarkers could be useful when saliva is used as diagnostic sample material (Gursoy et al. 2010).

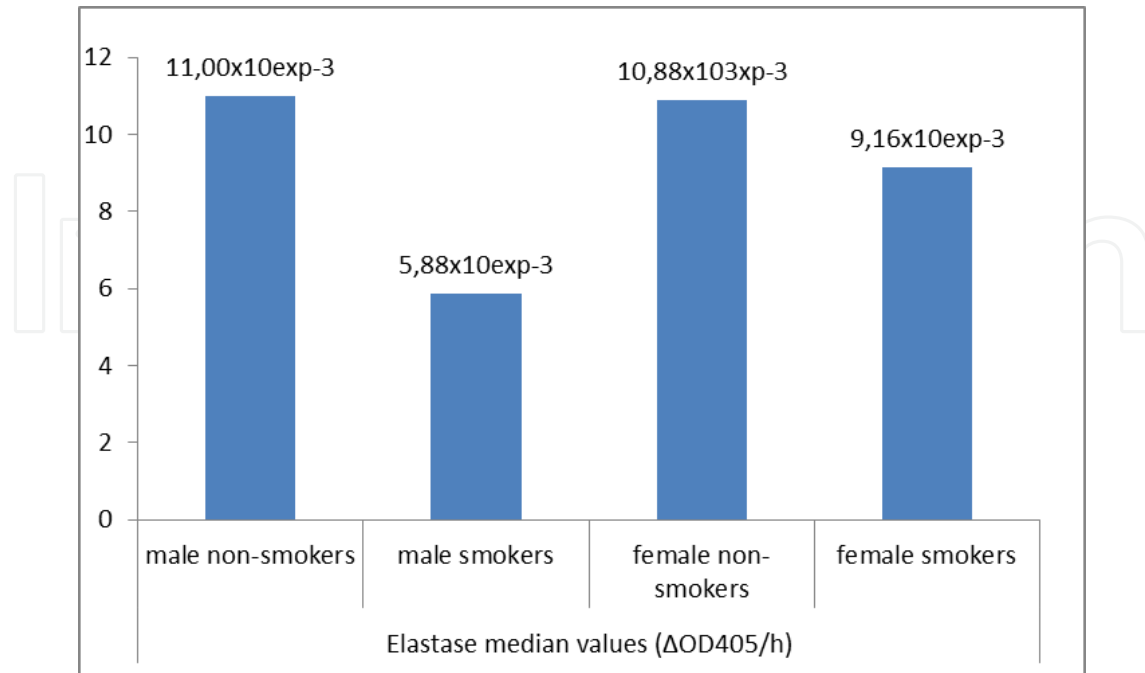


### 3.2. Smoking, MMP-8 and elastase levels in early periodontitis and their clinical relevance

In the study of adolescents Heikkinen et al. (2010) observed that smoking associated the lower levels of MMP-8 and PMN-leukocyte elastase (figure 1. and 2.) and the effect was strengthened by increased pack-years. However, 15% of adolescents in this birth cohort study seemed to have signs of early periodontitis (Heikkinen 2011). Salivary MMP-8 values were associated with BOP and suggestively with deep pockets in the non-smoking teenage boys. In adults MMP-8 has shown to be a key biomarker during early stages of periodontal diseases (Ramseier et al. 2009). Clinically smoking reduces the signs of gingivitis (Kumar and Faizuddin, 2011) masking periodontal diseases, and thus smokers have less observed signs of gingival inflammation, in adolescents as well as in adults, aggravating the diagnostics of periodontal disease. It is important that patients receive a proper periodontal diagnosing as part of their regular dental examination. Early diagnosis of periodontal disease could enable a successful therapeutic outcome, by reduction of etiologic factors such as smoking and by establishing periodontal therapy and maintenance protocol. Further, this might prevent the recurrence and progression of disease and reduce the incidence of tooth loss (Kumar et al. 2012). Recently Nhawator et al. (2014) demonstrated that neutrophil collagenase-2 lateral flow chair-side (point-of-care) immunoassay analysed from mouth rinse had a high sensitivity for at least two sites with BOP and two sites with periodontal pockets but a lower relationship for single-site pockets and BOP. Further studies are needed to find out the clinical relevance for this test as a screening tool in adolescents finding early periodontitis as well as for adults taking account the effect of confounders such as smoking into inflammatory biomarkers.



**Figure 1.** Salivary MMP-8 median values corresponding smoking and sex in adolescents. CI 95% for male non-smokers, male smokers, female non-smokers and female smokers are 135.08-220.20 ug/l, 86.20-173.22 ug/l, 145.16-215.33 ug/l, 136.72-230.68 ug/l, respectively.



**Figure 2.** Salivary elastase median values corresponding smoking and sex in adolescents. CI 95% for male non-smokers, male smokers, female non-smokers and female smokers are  $8.75-13.63 \times 10 \exp^{-3} \Delta OD405/h$ ,  $4.75-9.25 \times 10 \exp^{-3} \Delta OD405/h$ ,  $8.75-15.25 \times 10 \exp^{-3} \Delta OD405/h$ ,  $6.63-17.25 \times 10 \exp^{-3} \Delta OD405/h$ , respectively

### 3.3. Predictive value of oral fluid MMP-8

It should be noted that MMP-8 levels in oral fluid possess a predictive value (Sorsa et al. 2010, Munjal et al. 2007, Prescher et al. 2007, Kraft-Neumärker et al. 2012, Leppilahti et al. 2014a,b). In this context, periodontitis patients were examined and followed over a course of 12 months at 2 month intervals. In these patients it was possible to clearly differentiate “stable sites” from “unstable” sites.

- “Stable sites”: Improvement in pocket depth (PD) and attachment loss (AL) were continuously preserved after treatment, similarly the GCF MMP-8 values were and remained consistently low.
- “Unstable sites”: No improvement or only temporary improvement in PD and AL were found, in parallel GCF MMP-8 values only improved shortly after treatment, followed by an immediate re-increase in the MMP-8 values.

Furthermore, Reinhardt et al. (2010) and Leppilahti et al. (2014a) demonstrated that increases in GCF MMP-8 during the periodontal maintenance are associated with increased odds of subsequent periodontal attachment loss and compromised treatment outcome. Overall, these authors concluded that elevated biomarkers of inflammation and bone resorption identify patients vulnerable to progressive periodontitis.

### 3.4. Elastase, protease inhibitors and sICAM-1

Elastase is another important neutrophil originating proteolytic enzyme. Contradicting findings about elastase activity in GCF of smokers compared with non-smokers with periodontitis have been reported, however: significantly higher mean levels of neutrophil elastase activity in smokers' than non-smokers' sites with matching PD has been detected (Söder 1999), but on the other hand lower concentrations of functional elastase in smokers' than in non-smokers' GCF has also been found (Alavi et al. 1995). This finding led Alavi et al. (1995) to the hypothesis that smokers' neutrophils may release elastase prior to reaching the periodontal tissues for example during passing through the lungs, or possibly a greater proportion of the elastase is bound to substrate and remains undetected which may complicate the diagnostic value of GCF elastase in smokers.

Smoking possibly intervenes in the levels of protease inhibitors  $\alpha$ 2-macroglobulin ( $\alpha$ 2-MG) and  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT), which may be one mechanism by which smoking can affect the inflammatory process. In severe periodontal lesions of smokers significantly lower concentrations and total amounts of GCF  $\alpha$ 2-MG as well as significantly lower total amounts of  $\alpha$ 1-AT were found. These findings lead to conclusion that decreased local levels of these inhibitors may result in increased tissue damage due to increased activity of elastase and collagenase (Persson et al. 2001).

A soluble form of intercellular molecule-1 (sICAM-1) is known to be elevated in smokers' blood compared with non-smokers (Koundouros et al. 1996). Conversely, in smokers with periodontitis GCF sICAM-1 is significantly lower compared with non-smokers (Fraser et al. 2001). Based on this finding Fraser et al. (2001) hypothesised that sICAM-1 molecules possibly bind to sequestered neutrophils in periodontal microvasculature and provoke an inappropriate endogenous protease release contributing to periodontal destruction in the vicinity of the gingival microvasculature.

### 3.5. Cytokines

Bacterial products stimulate monocytes, macrophages and lymphocytes as well as resident fibroblasts and endothelial cells to secrete pro-inflammatory and immunoregulatory cytokines, which control cell growth and differentiation. Bacteria further stimulate chemokines and pro-inflammatory cytokines or subdue with anti-inflammatory cytokines and interferons the inflammation and regulate the development of the antimicrobial immunity in cooperation with antigen presenting cells (Julkunen et al. 2003). Smoking appears to affect normal balance of several cytokines, which are described as local hormones or cell-to-cell messengers. Especially the reduction of chemokines in smokers has been regarded to contribute to weakened neutrophil chemotaxis and migration to the site of inflammation in spite of the existing leukocytosis (Palmer et al. 2005).

In earlier studies increased levels of tumour necrosis factor (TNF)  $\alpha$  but decreased levels of IL-6 and IL-1 $\beta$  were detected in GCF of smoking periodontitis patients compared with non-smokers especially with tendency towards higher TNF- $\alpha$  levels in sites with an inferior treatment outcome (Boström et al. 1998a, 1999, 2000). Former smokers have also been reported



to exhibit significantly higher GCF levels of TNF- $\alpha$  than non-smokers (Boström et al. 1998b). Parallel effect of smoking has been detected on GCF IL-10 both prior to as well as after periodontal treatment compared with non-smokers (Goutoudi et al. 2004).

Smoking seems to decrease the mean levels of GCF IL-1 $\alpha$  concentrations significantly but does not affect mean total protein concentration (Petropoulos et al. 2004). In this study by Petropoulos et al. (2004) neutrophil numbers were not significantly different between smokers and non-smokers suggesting that the reduced IL-1 $\alpha$  concentration of smokers may be independent of any effect of smoking on neutrophil chemotaxis, and smoking may directly inhibit IL-1 $\alpha$  production. Thus GCF IL-1 $\alpha$  may be derived from the inflamed tissues rather than being locally produced by neutrophils in pocket.

Recently multiplex immunoassays have been used to analyse simultaneously multiple GCF cytokines. A comprehensive investigation by a multi-bead array assay facilitated the characterization of 22 GCF cytokines, which were studied with respect to possible alterations in host response caused by smoking (Tymkiw et al. 2011). Quantities of pro-inflammatory cytokines, chemokines and regulators of T-cells and NK cells were found to be affected by smoking. Healthy sites of smoking periodontitis patients showed significantly less IL-6 and IL-12 than similar sites of non-smoking patients. In addition to these, smokers' periodontitis sites showed also significantly lower quantity of IL-1 $\alpha$ . Of chemokines IL-8, IL-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP) -1 $\alpha$  and RANTES were detected in lower amounts both from healthy and diseased sites of smoking periodontitis patients compared with similar sites in non-smokers suggesting that low chemokine response leads to inability to recruit inflammatory and immune cells and further to ineffective defence. This may have a major role in the pathogenesis of periodontitis in smokers. Also IL-7 and IL-15, regulators of T-cells and NK cells, showed a decrease in smokers compared with non-smokers (Tymkiw et al. 2011).

However, another study also utilizing a multiplex immunoassay concluded that there were no correlations between GCF levels of MIP-1 $\alpha$  and RANTES and the smoking status suggesting that at the local level smoking is not a major determinant of the CC group chemokine concentrations in GCF, and that the determinant is the level of local inflammation (Haytural et al. 2014). Another contradicting finding was detected by analysing MCP-1 with enzyme linked immunosorbent assay (ELISA), where MCP levels in GCF were highest in smokers with periodontitis when compared with non-smoking periodontitis patients and healthy controls (Anil et al. 2013), showing the possible effect of the analyse method on the results.

### 3.6. sRANKL and OPG

Soluble receptor activator of nuclear factor  $\kappa$  B ligand (sRANKL), its cellular receptor RANK and osteoprotegerin (OPG), a protein, which binds to RANKL blocking its interaction with RANK, are the regulators of bone formation and resorption (Tang et al. 2009). Periodontitis patients compared with healthy controls exhibit higher expression of RANKL in gingival tissues and GCF, which associates especially with active sites (Vernal et al. 2004). RANKL:OPG ratios may be increased in GCF of periodontitis patients (Bostanci et al. 2007). In current and former smoking periodontitis patients GCF OPG concentrations were lower compared with

never smokers, and finding was opposite concerning the sRANKL concentration (Tang et al. 2009). Consequently, the sRANKL:OPG ratio also appeared to be higher in current and former smokers but the finding was not statistically significant. Interestingly, when pack-years were included in the analysis, OPG concentration decreased with increasing pack years and also the sRANKL:OPG ratio was significantly higher in the high pack-years group being significant also in the multivariate analysis (Tang et al. 2009). An increased lifetime exposure above a minimum threshold of cigarette smoking was required for this pattern. This finding is supported by earlier results where the combination of lipopolysaccharide and nicotine were shown to decrease OPG production in osteoblasts in a dose dependent manner (Tanaka et al. 2006) and where periodontal ligament fibroblasts and epithelial cells directly exposed to nicotine decreased their overall protein synthesis (Giannopoulou et al. 2001; Chang et al. 2002). This may lead to increased sRANKL:OPG ratio in smokers and further cause imbalanced tissue homeostasis and consequent tissue degradation (Tang et al. 2009).

#### **4. Systemic inflammation and salivary biomarkers**

Analyzing and utilization of inflammatory and disease specific biomarkers in saliva could offer an attractive solution for the diagnosis of different systemic diseases (Rathanayake et al. 2013b). The composition of saliva mainly originates from blood but in the salivary glands active transport and secretion mechanisms may change the saliva composition as the organic components of glandular specific saliva are derived from protein synthesis and are stored within the acinar cells (Kaufman & Lamster (2002), Malamud 1992). Nevertheless, saliva could be an alternative to blood as a biological fluid for analysis in diagnostic and prognosis purposes since the collection of saliva is non-invasive and is a plausible method. Systemic inflammation leads to the relief of pro-inflammatory mediators from immune cells, and the activation of the innate immune system. An increasing number of specific molecular markers for different conditions, such as cancer, cardiovascular disease (CVD), rheumatoid arthritis (RA), diabetes and human immunodeficiency virus has been identified (Boyle et al. 1994, Hu et al. 2008, Zhang et al. 2010).

##### **4.1. Cardiovascular disease**

High sensitive methods for biomarker detection have been developed since year 2000. There are certain biomarkers released due to a myocardial injury caused by myocardial ischemia and necrosis, such as cardiac troponins I (TnI) and T (TnT), creatine kinase-MB (CK-MB), total creatine kinase, myoglobin, and lactate dehydrogenase (Mueller et al. 2013, Tiwari et al. 2012). Analysis of cardiac TnI and TnT are considered as the golden standard for diagnosis of acute myocardial infarction (AMI) as they are tissue specific for the myocardium (Tiwari et al. 2012). There are few earlier publications that have revealed correlations between serum and salivary biomarkers of CVD (Mirzaii-Dizgah et al. 2012, Quillet-Morin et al. 2011). The Tn I levels reaches its peak within 10–14 hours followed to an AMI, and according to the previous studies Tn I levels could be detected in saliva within 24 hours of onset of AMI (Mirzaii-Dizgah & Riahi 2013). A bedside saliva-based Nano-Biochip test together with electrocardiogram

could provide prompt screening method for AMI patients in prehospital stage and the investigators of this study were able to detect elevated salivary levels of creatine kinase-MB, myoglobin, TnI and TnT, C-reactive proteins (CRP), TNF- $\alpha$ , MMP-9 and myeloperoxidase from AMI patients (Floriano et al. 2009).

In the study of Palm et al. (2013) on patients with acute ischemic stroke, systemic and local inflammatory markers were analysed of patients saliva. In this study, controls had enhanced levels of salivary MMP-8, MPO and IL-1 $\beta$  compared to the patients, since the control group was suffering from ongoing periodontal disease and the patients more often had evidence of end-stage periodontitis with edentulism and missing teeth. They also had higher levels of serum MMP-8 and MPO. Additional longitudinal studies are needed, however, to check the potential of salivary biomarkers associated in ischemic stroke.

#### 4.2. Diabetes

There are a few studies concerning the detection of inflammatory biomarkers in saliva of patients with diabetes. Goodson and co-authors reported that in a child population unstimulated saliva samples were analysed and the salivary levels of CRP, insulin and leptin were remarkably higher in obese children compared with healthy normal weight children (Goodson et al. 2014). In a cross sectional study on 451 patients elevated salivary levels of MMP-8 were found among diabetes patients (Rathnayake et al. 2013). Salivary N-acetyl- $\beta$ -D-hexosaminidase (HEX) which is associated with type I diabetes was found to be significantly increased in children with type 1 diabetes compared with healthy children (Zalewska-Szajda et al. 2013a).

#### 4.3. Rheumatoid arthritis

The disease pattern of RA is similar to periodontal disease. Systemic inflammatory biomarkers from different chronic inflammatory conditions, such as RA could thus appear in saliva. There are few studies in this area, but when conducting an exploration of inflammatory biomarkers in RA patients, the periodontal status and the anti-TNF- $\alpha$  therapy taken by these patients need to take in to consideration. Salivary IL-1 $\beta$  was found to be significantly higher in the RA patients who were not on anti-TNF- $\alpha$  therapy compared with RA patients receiving anti-TNF- $\alpha$  therapy (Zalewska-Szajda et al. 2013b). Salivary exoglycosidases for detection of salivary gland involvement in RA patients were studied, in xerostomic RA group salivary  $\beta$ -glucuronidase was found to be significantly higher compared with healthy controls but the activity of salivary N-acetyl- $\beta$ -hexosaminidase and  $\beta$ -glucuronidase was significantly lower than in xerostomic hyposalivary RA patients (Zalewska-Szajda et al. 2013b).

#### 4.4. Cancer

To use salivary biomarkers to detect on / monitor all types of cancer is a growing research field in salivary diagnostics. The most common malignant neoplasm of the oral cavity is oral squamous cell carcinoma (OSCC). Patients with OSCC indicated that a specific marker of oxidative stress, malondialdehyde (MDA) in saliva was a better diagnostic tool as MDA in blood (Rasool et al. 2014). Salivary IL-8 levels seem to be higher in patients who had experi-

enced tumour diseases (Rathnayake et al. 2013a,b). To detect head and neck squamous cell carcinoma (HNSCC) microRNAs (miRNAs) of saliva was used, and the results showed that miR-9, miR-134 and miR-191 were differentially expressed between saliva from HNSCC patients and healthy controls. Additionally, the authors suggested that these saliva-derived miRNAs may serve as novel biomarkers to reliably detect HNSCC (Salazar et al. 2014).

There are number of cytokines and chemokins involved in the cancer progression, such as interferon-gamma (IFN- $\gamma$ ), TNF- $\alpha$ , IL-1 $\beta$ , transforming growth factor-beta-1 (TGF- $\beta$ 1), epidermal growth factor (EGF), IL-6 and -8, vascular endothelial growth factor (VEGF), interleukins-4 and -10, tumour necrosis factor (TNF) and endothelin. Saliva based testing of these biomarkers is promising depending on the methods of analysis (Prasad & McCullough 2013). About 5 % of all cancers of the head and neck are salivary gland carcinomas (SGCs). Thus there is a need to develop new molecular biomarkers for early diagnosis and to improve the diagnosis of SGCs. Further research in this is required.

To identify disease specific molecular biomarkers in whole saliva is challenging. There are certain biomarkers found in saliva of high sensitivity and specificity, particularly in oral diseases, such as periodontal disease and oral cancer. There are factors that have an influence for the expression and release of biomarkers, such as their intracellular location, the size of the proteins, and the characteristics of the local biological fluid flow. The type of saliva used for diagnostic purpose to detect systemic conditions has an impact. In this regard unstimulated saliva reveals more information than stimulated saliva since unstimulated saliva contains higher concentrations of diagnostic biomarkers. High sensitivity and sophisticated methods and techniques are required for valuable outcome of the analyses of saliva samples.

## 5. Conclusion remarks

Inflammatory saliva and GCF biomarkers can be used as an aid in periodontal diagnostics, but there is a need to define the range of physiological levels and cutoff for pathological bursts of periodontitis progression. However, using just one definite cutoff point or merely one biomarker is not rational for adults or adolescents. Adolescence might have certain characteristics with different cutoff points compared to adults. The clinical use of salivary biomarkers to identify systemic conditions is another interesting area for developing non-invasive screening and diagnostic procedure. This might be the main goal for saliva research but in this regard it is important to consider the influence of oral health conditions which may confound the utility of the biomarkers. Modifying factors, such as smoking and pregnancy also should be taken into account when interpreting the results of the oral fluid inflammatory biomarkers.

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