Systemic effects of initial periodontal therapy with or without adjunctive systemic antibiotics

ALMAGHLOUTH, Adnan Ali

Abstract

Les sujets atteints de parodontite non traitée peuvent montrer des pics élevés pendant plusieurs marqueurs inflammatoires dans le sérum simultanément. Traitement parodontal non chirurgical avec ou sans antibiotiques réduit la plupart de ces niveaux de pointe.

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"Systemic effects of initial periodontal therapy with or without adjunctive systemic antibiotics"

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présentée à la Faculté de médecine
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par

Adnan Ali Saleh ALMAGHLOUTH

de

Riyad, Royaume d'Arabie Saoudite

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Abstract

Background and Objective: Some subjects with untreated periodontitis exhibit elevated levels of distinct inflammatory markers in serum. The aim of the study was to assess whether non-surgical periodontal therapy changes the levels of these markers and lowers these peaks.

Methods: 40 periodontally diseased subjects received non-surgical periodontal therapy (full-mouth scaling and root planing within 48 h) with either adjunctive systemic amoxicillin and metronidazole (n=19) or placebo (n=21). Serum samples, obtained at baseline (BL) and 3 months after treatment (M3), were evaluated for 15 cytokines and 9 acute-phase proteins using the Bio-Plex bead array multianalyte detection system. For each analyte, peak values were defined as >mean+2SD in 40 periodontally healthy control subjects. Proportions were compared using Fisher’s exact test.

Results: At M3, a significantly better primary clinical outcome (persisting pockets >4 mm with bleeding on probing) was obtained in patients treated with scaling and root planing plus antibiotics compared to those receiving placebo (3.3±5.1 v.s. 6.8±7.8 pockets per patient, p<0.05). The levels of cytokines and acute phase proteins of periodontitis patients were usually below the mean+2SD threshold of healthy controls. However, values above threshold were found in some individuals. 11 patients showed a peak value of one analyte, 7 patients showed two peaks. In the remaining 12 patients, between 3 and 10 analytes showed peak values. Therapy greatly reduced the number of subjects with 4 or more peaks (BL: 11 subjects, M3: 1 subject, p=0.003). With regards to the reduction of peaks, no specific benefit of adjunctive antibiotics could be seen.

Conclusion: Subjects with untreated periodontitis may show high peaks for several inflammatory markers in serum simultaneously. Non-surgical periodontal treatment with or without antibiotics reduced most of these peak levels.
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List of abbreviations

α2M:       Alpha-2 macroglobulin
b-FGF      Basic fibroblast growth factor
BL         Baseline
BOP        Bleeding on probing
CRP        C-reactive Protein
DM         Diabetes mellitus
ELISA      Enzyme-linked immunosorbent assay
GCF        Gingival crevicular fluid
GI         Gingival Index
G-CSF      Granulocyte colony stimulating factor
GM-CSF     Granulocyte macrophage colony stimulating factor
Hp         Haptoglobin
IFN-γ      Interferon-gamma
IL-1β       Interleukin-1 beta
IL-1ra      Interleukin-1 receptor antagonist
IL-4        Interleukin-4
IL-6        Interleukin-6
IL-8        Interleukin-8
IL-10       Interleukin-10
IL-12       Interleukin-12
IL-17       Interleukin-17
MIP-1β      Macrophage inflammatory protein-beta
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>mean+2SD</td>
<td>Mean plus two standard deviations</td>
</tr>
<tr>
<td>M3</td>
<td>Month 3</td>
</tr>
<tr>
<td>MBAA</td>
<td>Multiplex Bead Array Assay</td>
</tr>
<tr>
<td>PD</td>
<td>Probing depth</td>
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<tr>
<td>PCT</td>
<td>Procalcitonin</td>
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<tr>
<td>REC</td>
<td>Recession</td>
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<tr>
<td>SRP</td>
<td>Scaling and root planing</td>
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<tr>
<td>SAA</td>
<td>Serum amyloid A</td>
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<tr>
<td>SAP</td>
<td>Serum amyloid P</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue plasminogen activator</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Introduction

Background

Periodontitis is an inflammatory disease of the periodontium characterized by inflammation of the gingiva and adjacent attachment apparatus, illustrated by loss of clinical attachment due to destruction of the periodontal ligament and loss of adjacent supporting bone. Periodontitis is an important public health problem. In the United States alone, the prevalence of total periodontitis (i.e., the sum of mild, moderate, and severe periodontitis according to American Academy of Periodontology definitions) was 47% of the total population. The goal of periodontal therapy is to preserve the natural dentition and maintain periodontal health, comfort, aesthetics and function.

Recent data have suggested that severe periodontitis is associated with elevated inflammatory markers in otherwise healthy populations. These include matrix metalloproteinases, a variety of cytokines and chemokines, inflammatory markers, antiphospholipid antibodies, and antibodies to periodontal pathogens. Subjects with periodontitis have increased serum levels of C-reactive protein (CRP), hyperfibrinogenemia, and increased serum levels of Interleukin (IL)-1 and -6, as compared to periodontally healthy subjects. Despite the fact that poor periodontal health is linked to higher levels of several systemic markers, only few studies have tried to elucidate whether successful periodontal treatment can reduce the levels of these serological markers. Changes were observed after periodontal treatment in serum IL-6, CRP, total and low-density lipoprotein cholesterol, as well as on biomarkers of vascular health. Technological developments have made it possible to detect and quantify a range of various biological markers in relatively small fluid specimens simultaneously. A high-throughput bead-based suspension array immunoassay system can assess up to 100 analytes in a sample volume of 25 to 50 µL. The quantification of multiple markers in one single same sample can provide information on interactions that are inaccessible by studying single markers individually.

A previous randomized clinical trial performed by our group demonstrated that systemic metronidazole and amoxicillin significantly improved the 6 months clinical outcomes of full mouth non-surgical mechanical periodontal treatment, thus significantly reducing the need for additional surgical therapy. A precautionary, restrictive attitude toward using antibiotics is however generally recommended. To limit the development of microbial antibiotic resistance in general, and to avoid the risk
of unwanted systemic effects of antibiotics it is postulated that mechanical therapy should be exploited to its limits before a decision is made to administer an antibiotic. With regards to the latter, it was noted in the study cited above that the number of subjects complaining about gastro-intestinal problems, notably diarrhea, was indeed higher in the test than the placebo group. On the other hand, tooth loss and suppuration despite therapy were exclusively noted in subjects of the placebo group. The frequency and potential consequences of unwanted systemic effects of the antibiotics have to be put into perspective with potential health consequences of not quickly suppressing a periodontal infection. Intervenational studies showing systemic effects of successful periodontal therapy support this hypothesis.

**Inflammatory markers in medicine and periodontology**

Inflammation is a protective mechanism by the body to remove the injurious stimuli and to initiate the healing process. Inflammation can be classified into either acute or chronic. Acute inflammation is the primary response of the body to harmful stimuli and is accomplished by the increased movement of plasma and leukocytes from the blood into the injured tissues. A series of biochemical events propagates the inflammatory reaction, involving the local vascular system, the immune system, and various cells within the injured tissue. Chronic inflammation develops following the prolongation of the inflammation. It leads to a significant shift in the type/quality of cells present at the site of the inflammation. Characteristic features include destruction and healing of the tissue affected by the inflammatory process.

Periodontitis, as any other inflammatory disease in the body, results in fluctuating/disturbed inflammatory proteins that reflect the inflammatory state. These proteins/markers may be divided into two types: ones that derived directly from periodontopathic pathogens such as bacterial proteins, toxins, or proteases. The other ones originate in the defense mechanisms by the host and include antibodies to periodontal pathogens, acute phase proteins, chemokines and cytokines, matrix metalloproteinases and their inhibitors.

In the following section the biological role of selected inflammatory markers in relationship with periodontitis will be reviewed.
The interleukin-1 receptor antagonist (IL-1RA)

This is a member of the interleukin 1 cytokine family. Many types of cells including immune cells, epithelial cells, and adipocytes secrete IL-1RA. It is a natural inhibitor of the pro-inflammatory effect of Interleukin 1β (IL-1β).\(^\text{14}\) IL-1RA inhibits the activities of interleukin 1α (IL-1α) and IL-1β, and modulates a variety of interleukin 1 related immune and inflammatory responses.

Clinical significance of IL-1RA

An association between gene polymorphisms of this protein and increased risk of osteoporotic fracture \(^\text{15}\) and gastric cancer \(^\text{16}\) has been reported. Its role in oral disease is not clear. Experimental studies have shown that IL-1RA may interfere with orthodontic tooth movement, probably by its anti-inflammatory activity \(^\text{17}\).

The interleukin-1 beta (IL-1β)

IL-1β is another member of the interleukin 1 cytokine family. It is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). IL-1β is an important mediator of the inflammatory response, and is involved in different cellular activities, including cell proliferation, differentiation, and apoptosis \(^\text{18}\).

Clinical significance of IL-1β

In medicine, polymorphisms of this protein have been linked to susceptibility to schizophrenia \(^\text{19}\). In the dental and periodontal literature in particular, the contribution of this cytokine to periodontitis has been shown in several studies, and a recent meta-analysis concluded that IL-1β and IL-1α genetic variations are significant contributors to chronic periodontitis in whites \(^\text{20}\). Studies on gingival crevicular fluid (GCF) documented the ability of the periodontal therapy to lower the level of IL-1β and improve the clinical condition of the disease \(^\text{21}\) \(^\text{22}\) \(^\text{23}\).

The interleukin-4 (IL-4)

This cytokine induces differentiation of naive helper T cells (Th0 cells) to Th2 cells. Upon activation by IL-4, Th2 cells subsequently produce additional IL-4 \(^\text{24}\). This cytokine has multiple biological roles, including the stimulation of activated B-cell and T-cell proliferation, and the differentiation of B cells into Plasma Cells. IL-4 induces B-cell class
switching to IgE and up-regulates major histocompatibility complex (MHC) class II production. IL-4 decreases the production of Th1 cells, macrophages, Interferon-γ (IFN-γ), and IL-12 25.

Clinical significance of IL-4

Overproduction of IL-4 has been associated with allergies 25. Moreover, the presence of IL-4 in extravascular tissues promotes alternative activation of macrophages into repair macrophages M2 cells and inhibits classical activation of macrophages into M1 cells. An elevated level of macrophages M2 is coupled with secretion of IL-10 and Transforming growth factor beta (TGF-β) that result in a diminution of pathological inflammation. Therefore, the release of arginase, proline, polyamines, and TGF-β by the activated M2 cell will result in wound repair and fibrosis 26. IL-4 polymorphism has been linked to the development of chronic periodontitis in some populations 27 28. Serum levels of this marker remained unchanged after conventional periodontal therapy alone, i.e., without adjunctive antibiotic treatment. 29 30.

The interleukin-6 (IL-6)

IL-6 is secreted by T cells and macrophages. IL-6 is a cytokine that provokes a broad range of cellular and physiological responses, including the immune response, inflammation, hematopoiesis, and oncogenesis by regulating cell growth, gene activation, proliferation, survival, and differentiation 31.

Clinical significance of IL-6

IL-6 has been linked with diseases such as diabetes 32, atherosclerosis 33, depression 34, Alzheimer’s disease 35, systemic lupus erythematosus 36, and rheumatoid arthritis 37. According to Laine et al. 2010, there is growing evidence that polymorphisms in IL6 and other proteins like IL1, IL10, vitamin D receptor, and CD14 genes may be associated with chronic periodontitis in certain populations 38. Elevated serum levels of IL-6 were significantly reduced following successful periodontal treatment 10 39. However, the same group concluded in a recent systematic review that this effect is negligible and will need to be proven in better-designed clinical trials 40.

The interleukin-8 (IL-8)

IL-8 is a member of the chemokine family. It has been identified as a neutrophil chemotactic factor 41 42 and is produced by various types of cells upon stimulation with
inflammatory stimuli including macrophages and other cell types such as epithelial cells. It is also synthesized by endothelial cells, which store IL-8 in their storage vesicles, the Weibel-Palade bodies. Besides its effect on neutrophils, it exhibits chemotactic activities against T lymphocytes and basophils. In addition, IL-8 induces neutrophils to release lysosomal enzymes, and to adhere to unstimulated endothelial cells.

**Clinical significance of IL-8**

Elevated IL-8 levels have been documented in several diseases including rheumatoid arthritis, adult respiratory distress syndrome, and chronic and aggressive periodontitis. A recent study showed that short-term nonsurgical therapy resulted in a significant improvement in periodontal indices and in a marked decrease of IL-8 GCF levels even though no significant correlations were found between clinical parameters and amounts of humoral factors after the therapy.

**The interleukin-10 (IL-10)**

IL-10 is an anti-inflammatory cytokine. It is also called human cytokine synthesis inhibitory factor. This cytokine is initially produced by monocytes and, to a lesser extent, lymphocytes, namely type 2 helper cells (Th2), mastocytes, and in a certain subset of activated T cells and B cells.

**Clinical significance of IL-10**

Besides the fact that IL-10 may be classified as a Th2-type cytokine, it has been demonstrated that it suppress a wide range of inflammatory responses and is known to be an important factor in maintaining homeostasis of overall immune responses. A recent meta-analysis found a statistically significant association of IL-10 -819 (-824) C>T and IL-10 -592 (-597) C>A polymorphisms in Caucasians, which may confer a relative increase in the risk for chronic periodontitis. It is still unclear if periodontal therapy can influence the level of this cytokine. In one study, scaling and root planning (SRP) with or without amoxicillin and metronidazole resulted in an increase in GCF levels of IL-10 and a reduction of IL-1β. Another study showed GCF IL-10 levels were similar in all treated pockets and did not change after periodontal therapy.

**The interleukin-12 (IL-12)**

IL-12 is a cytokine that is naturally produced by dendritic cells, macrophages and human B-lymphoblastoid cells in response to antigenic stimulation. It exerts
immunoregulatory effects on natural killer cells and T cells. IL-12 plays an important role in the initiation and maintenance of Th1 responses, especially in the induction of adaptive cellular immunity 59 60.

**Clinical significance of IL-12**

IL-12 is related to the differentiation of T H1 cells, and there is evidence that Th cells are directly involved in the progression of apical periodontal lesions and bone resorption. Another fact is that T H1 cells respond to macrophage-derived IL-12, releasing IFN-γ and suppressing T H2 cytokines, and hence favoring infection-induced bone loss. Thus, the IL-12-IFN-γ pathway may contribute to the progress of apical periodontal lesions due to its proinflammatory actions 61. On the other hand, in a study on mice bred to be allergic to peanuts, IL-12 has been shown not to be present, suggesting that the cytokine normally stops allergies to food developing. It is unknown whether the results found in mice are as profound in humans 62.

**The interleukin-17 (IL-17)**

IL-17 is a cytokine that acts as a potent mediator in delayed-type reactions by increasing chemokine production in various tissues to recruit monocytes and neutrophils to the site of inflammation, similar to IFN-γ. IL-17 is produced by T-helper cells and is induced by IL-23, which result in destructive tissue damage in delayed-type reactions 63. IL-7 acts synergistically with tumor necrosis factor (TNF-α) and IL-1 64 65.

**Clinical significance of IL-17**

IL-17 has pro-inflammatory effects, causing bone resorption and inflammation in rheumatoid arthritis by inducing IL-1β, TNF-α and IL-6. IL-17 also stimulates chemokine release (IL-8) and induces expression of various matrix metalloproteinases (MMPs). It also activates cyclooxygenase-2 (COX-2) and induces expression of Receptor activator of nuclear factor kappa-B ligand (RANKL) in osteoblasts, leading to bone resorption 66. A recent study confirmed the association of IL-17A -197A allele with increased risk for chronic periodontitis 67. Periodontal therapy yielded inconsistent results between GCF and serum levels of IL-17. One study showed elevated GCF levels of this marker following nonsurgical therapy, even without a difference between periodontitis in smokers and non-smokers 68. In addition, GCF levels of this marker were shown to be elevated after surgical debridement compared to nonsurgical therapy. However, this study was on periodontitis patients with type-2 diabetes 69. When serum levels were
analyzed, IL-17 and IFN-γ levels were reduced at six months following periodontal therapy but only in generalized aggressive periodontitis patients.30

**Basic fibroblast growth factor (b-FGF/FGF-2)**

b-FGF is synthesized and secreted by human adipocytes. In normal tissue, it is present in basement membranes and in the subendothelial extracellular matrix of blood vessels. It stays membrane-bound as long as there is no signal peptide.70

**Clinical significance of b-FGF**

Human diseases that are known to be caused by over-activity of FGF due to mutations in the genes encoding FGF receptors 1–3 include bone and cartilage diseases such as craniosynostosis, achondroplasia and hypochondroplasia.7172 Conversely, due to its ability to facilitate major reactions necessary for revascularization, b-FGF may have a promising role in inducing periodontal repair in guided tissue regeneration. In a phase II clinical trial a significant increase in alveolar bone height was seen in patients treated with gel containing 0.3% FGF-2 (Figure 1).71

**Figure 1**

Changes in alveolar bone height over time in 74 patients with two- or three-walled periodontal defects: group P, placebo; group L, 0.03% FGF-2; group M, 0.1% FGF-2; group H, 0.3% FGF-2. From Murakami et al.71
Granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF)

Colony-stimulating factors are specific hematopoietic factors needed for bone marrow progenitor cells to form mature blood cells. G-CSF stimulates the development of neutrophils, eosinophils, and basophils, whereas GM-CSF stimulates the generation of cells belonging to the monocyte/macrophage lineage. In addition, both G-CSF and GM-CSF enhance the function of peripheral neutrophils, including those in mucosal tissues. GM-CSF has activity on the proliferation of keratinocytes, and animal studies suggest that it enhances wound healing 73.

Clinical significance of G-CSF and GM-CSF

Direct actions of colony-stimulating factors on peripheral cells as well as a temporal relationship of healing of mucositis and bone marrow recovery have been the rationale for numerous clinical studies testing G-CSF and GM-CSF for the prevention and treatment of oral mucositis 73.

This cytokine has been shown to prolong the persistence of neutrophils in gingival tissues from chronic periodontitis subjects by reducing their apoptosis 74. In addition, the reduction in GCF-levels of GM-CSF after periodontal therapy implicates this cytokine in the pathogenesis of generalized aggressive periodontitis 21.

Interferon-gamma (IFN-γ)

IFN-γ is a cytokine that is important for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control. Aberrant IFN-γ expression is associated with a number of autoinflammatory and autoimmune diseases. The importance of IFN-γ in the immune system is attributed to its ability to inhibit viral replication directly, and to its immunostimulatory and immunomodulatory effects. IFN-γ is produced predominantly by natural killer and natural killer T cells as part of the innate immune response, and by CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops 75 76.

Clinical significance of IFN-γ

The expression of IFN-γ is noteworthy, because of not only its elevated transcriptional and translational expression in inflamed gingival tissues and GCF but also its association with advanced periodontal disease and disease progression 77. In a large molecular
epidemiologic study with 6768 community-based subjects, Offenbacher et al. 2007, reported a significant increase in the GCF concentration of IFN-γ in those subjects with deep periodontal pockets and severe gingival bleeding as compared with subjects with probing depth (PD) of <3 mm. A high level of Th1 cytokines was found in the GCF of patients with extremely severe (terminal stage) periodontitis, including a 10-fold increase in the concentration of IFN-γ when compared with the Th2 mediators IL-4 and IL-6. The presence of high concentration of IFN-γ is shown to enhance the phagocytic activity of monocytes and neutrophils, which helps containment of infection. IFN-γ upregulates monocyteic response to lipopolysaccharides, which results in elevated monocyteic secretion of proinflammatory molecules, such as prostaglandin E2 (PGE₂), IL-1β and TNF-α, all of which play important roles in bone loss and the disintegration of soft tissue in the periodontium. The influence of periodontal therapy on IFN-γ GCF levels is unclear. SRP, with or without adjunctive antibiotics did not change its levels. In another trial, periodontal treatment was evaluated in patients with aggressive periodontitis. Here, GCF and saliva levels of IFN-γ, among other markers, were shown to decrease following therapy. The authors concluded that cytokine levels in GCF and saliva correlated well with clinical parameters.

**Macrophage Inflammatory Protein 1 beta (MIP-1β)**

Macrophage inflammatory proteins are chemokines that exist in two major forms, MIP-1α and MIP-1β, and are now officially named CCL3 and CCL4, respectively. Both are major factors produced by macrophages after stimulation with bacterial endotoxins. They activate human granulocytes (neutrophils, eosinophils and basophils), which can lead to acute neutrophilic inflammation. They also induce the synthesis and release of other pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α from fibroblasts and macrophages. Besides macrophages, other cells including dendritic cells, and lymphocytes produce them. CCL4 is a major HIV-suppressive factor produced by CD8+ T cells.

**Clinical importance of MIP-1β/CCL4**

In chronic hepatitis C, intrahepatic and peripheral blood levels of MIP-1β/CCL4 reflect viral persistence. In subjects with chronic periodontitis, a study showed significantly higher amounts of GCF MIP-1, together with a range of other pro-inflammatory cytokines. The author also found that smokers displayed decreased amounts of MIP-1 and other markers when compared to non-smokers. Serum levels of MIP-1β in the
order of 100 pg/ml have been measured in healthy subjects and highly variable levels up to 1000 pg/ml have been reported from patients with infectious diseases such as borreliosis.

**Vascular endothelial growth factor (VEGF)**

VEGF-A, -C, and -D are important signaling proteins produced by cells and involved in angiogenesis and lymphangiogenesis (the growth of blood/lymphatic vessels, respectively, from preexisting vasculature). These VEGFs exert their biologic activities via specific tyrosine kinase receptors. VEGF-A binds to vascular endothelial growth factor receptor (VEGFR)-1 and VEGFR-2 and acts primarily as angiogenic factor, whereas VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3 and are mainly lymphangiogenic factors. In bone destructive pathologic processes like cancer metastasis to bone, multiple myeloma, and rheumatoid arthritis, the VEGF family represents a link between growth of blood vessels (angiogenesis) and bone turnover, and the presence of VEGFs and their receptors on osteoblasts and osteoclasts gives additional evidence that the VEGF-VEGFR axis is involved in bone resorption processes. Apart from their major effects on vasculature, VEGFs have an involvement in differentiation and maturation of dendritic cells and of T- and B-lymphocytes.

**Clinical importance of VEGF**

In healthy subjects VEGF has been found at levels ranging from 60-700 pg/ml in serum and 0-115 pg/ml in plasma respectively. In diseases associated with an increase of angiogenesis, serum VEGF levels are highly variable. The median concentration in idiopathic myelofibrosis with myeloid metaplasia was 1200 ng/ml. VEGF-D serum levels have been found significantly elevated in patients with angiosarcoma. A recent study emphasized the integral role of VEGF and TGF-β1 in the evolution of the immune response, which in turn influences the outcome of periodontal disease establishment. A recent study found GCF and serum VEGF levels increased progressively with periodontal disease severity and decreased after treatment.

**Tumor necrosis factor-alpha (TNF-α)**

TNF-α is a member of a group of peptide mediators comprising at least 19 cytokines, including lymphotoxin-α, Fas ligand, and CD40 ligand. TNF-α has important pro-inflammatory properties, which play crucial roles in the innate and adaptive immunity, cell proliferation, and apoptotic processes. The cytokine is produced by different types
of cells, including macrophages, monocytes, T-cells, smooth muscle cells, adipocytes, and fibroblasts 97.

Pro-inflammatory effects of TNF-α mainly result from its effects on endothelial cells and their interaction with leucocytes. TNF-α causes increased expression of Intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1) and E-selectin, which, together with release of chemokines (e.g. IL-8), results in recruitment of leucocytes. TNF-α also induces expression of COX-2 in endothelial cells, leading to vasodilatation and increased vascular permeability 66.

Clinical importance of TNF-α

TNF-α is of special interest for the understanding of immune responses in relation to a linkage between rheumatoid arthritis and periodontitis 101. TNF-α is released in response to lipopolysaccharide and other bacterial byproducts. A local increase in TNF-α concentration results in heat, swelling, redness, pain, and loss of function. Elevated serum levels of TNF-α induce production of CRP and promote the expression of adhesion molecules on endothelial cells, allowing neutrophil diapedesis and inducing IL-1 activation. Within the cytokine cascade, IL-1 stimulates synoviocytes to produce MMPs (stromelysin) with activation of collagenase, resulting in cartilage destruction. IL-1 is a chemoattractant facilitating the migration of polymorphonuclear cells into the synovial tissues. High levels of IL-1 also cause increased production of nitric oxide killing of chondrocytes. IL-1 regulates NFκB-osteoprotegerin-RANKL and induces osteoclast activation. These inflammatory processes result in osteolysis in both rheumatoid arthritis and periodontitis.

After treatment of chronic gingivitis and periodontitis, GCF levels of TNF-α decreased, reflecting the improvements in clinical parameters following non-surgical mechanical treatment alone 81, or when combined with adjunctive Nd-YAG laser 98. Saliva levels of this marker corresponded to the levels in GCF 81 99. However, the effect of periodontal therapy on the serum levels of TNF-α seems to be different. Unlike IL-6 and CRP, non-surgical mechanical treatment alone did not result in a significant decrease of serum TNF-α levels in chronic periodontitis patients 100.

Due to the fact that treatment with anti-TNF-α medication is commonly used to control for the inflammatory process in rheumatoid arthritis, such therapy may also be relevant for the management of periodontitis 101.
**Alpha-2-Macroglobulin (α2M)**

α2M is a large plasma protein found in the blood. It is produced by the liver, and is a major component of the alpha-2 band in protein electrophoresis. Besides the liver as a source, macrophages, fibroblasts, and adrenocortical cells produce this protein.

α2M is able to inactivate an enormous variety of proteinases (including serine-, cysteine-, aspartic- and metalloproteinases). It functions as an inhibitor of fibrinolysis by inhibiting plasmin and kallikrein. In addition, it inhibits coagulation by inhibiting thrombin.

**Clinical importance of α2M**

This acute phase protein is delivered to the blood stream as an early response to different stimuli, such as tissue injury, inflammation, and infection.

In dental medicine research, α2M has been used as a biological marker reflecting disease status before and after dental therapy. In a split mouth study, Knöfler and coworkers compared the influence of topical metronidazole gel application and SRP on GCF components. The variables selected were aspartate aminotransferase and total/transformed α2M. The authors concluded that α2M reflects clinical changes better than aminotransferase and that metronidazole and SRP have the same influence on clinical outcome and biochemical variables in the GCF.

**Haptoglobin (Hp)**

Hp is an acute-phase protein whose transcription is increased in response to inflammatory stimuli. Hp has several biological functions, but it is best known as a hemoglobin (Hb) binding protein. Hp/Hb complex formation serves to reduce the oxidative damage, which would otherwise occur between heme-derived iron and surrounding plasma or tissue proteins and lipids. Binding of Hb to Hp is especially important during hemolysis, when free Hb levels in the plasma increase, leading to Hb accumulation in the kidney and iron loss in the urine. By binding to Hp, the Hp/Hb complex is formed and cleared by receptor-mediated endocytosis in the liver. In this manner, the iron is recycled and renal damage is prevented.
Clinical importance Hp

Polymorphism in Hp has been linked to diabetic nephropathy, the incidence of coronary artery disease in type-1 diabetes and Crohn’s disease. In periodontology, it is unclear if this polymorphism plays a role in the susceptibility to chronic periodontitis or peri-implantitis. In a study from our group, the GCF level of Hp was measured before and after the therapy among eight other acute phase proteins and 13 cytokines. The treatment was photodynamic therapy, diode soft laser therapy, or SRP alone. Hp levels were significantly higher at 6 months than at baseline and no significant differences were observed among the three treatment modalities at any time point for any biochemical parameter.

C-reactive protein (CRP)

CRP is an acute-phase protein found in the blood, the levels of which rise in response to inflammation. It is synthesized by the liver in response to factors released by macrophages and fat cells (adipocytes). It binds to phosphocholine, expressed on the surface of dead or dying cells (and some types of bacteria), in order to activate the complement system. CRP is a member of the class of acute-phase reactants, as its levels rise dramatically during inflammatory processes occurring in the body. During the inflammation, its level increases gradually. This is thought to be due to a rise in the plasma concentration of IL-6, which is produced predominantly by macrophages as well as adipocytes. CRP rises up to 50,000-fold in acute inflammation, such as infection. It rises above normal limits within 6 hours, and peaks at 48 hours. Its half-life is constant, and therefore its level is mainly determined by the rate of production and hence the severity of the precipitating cause. Serum amyloid A is a related acute-phase protein that responds rapidly in similar circumstances.

Clinical importance of CRP

A high CRP level is an independent risk factor for atherosclerotic disease. Patients with high CRP concentrations are more likely to develop stroke, myocardial infarction, and severe peripheral vascular disease. Normal concentration in healthy human serum is usually lower than 10 µg/ml. During inflammation, the level may rise in mild inflammation and viral infections up to 40 µg/ml, bacterial infection up to 200 µg/ml, severe bacterial infections and burns to more than 200 µg/ml. Several studies have shown increased levels of serum CRP in subjects with generalized aggressive periodontitis and chronic periodontitis as compared with the controls.
Moreover, the combination of chronic infections like periodontitis with elevated CRP is associated with higher chronic heart diseases. Periodontal treatment resulted in significant reductions of serum and plasma levels of this marker. In a recent systematic review, a positive effect of periodontal therapy was found in reducing serum CRP levels and improving endothelial function.

**Amyloid P component, serum (SAP)**

SAP is a plasma glycoprotein. It is a member of the pentraxins family, characterized by calcium dependent ligand binding and a distinctive structure similar to that of the legume lectins. The human SAP has 51% sequence homology with CRP, although both proteins are functionally distinct. Both SAP and CRP are conserved throughout vertebrate evolution. Like CRP, SAP is synthesized and secreted in hepatocytes and has a half-life of approximately 24 hours. The biological role of SAP is only partly known. It binds in a calcium-dependent way to molecular arrays such as DNA, chromatin, histones, and phosphoethanolamine-containing membranes, suggesting that it has a function in clearance of late apoptotic cells.

**Clinical importance of SAP**

According to Koenig (2007), SAP is different to CRP in the sense that it is only mildly affected during acute or chronic inflammation and serum concentrations remain close to the normal range (10 to 50 mg/L).

SAP is a universal constituent of amyloid deposits that are characteristic of systemic amyloidosis, including cerebral amyloid in Alzheimer disease and in type-2 diabetes mellitus (DM-2). SAP has also been claimed to be present in atherosclerotic plaque. However, grouping these different diseases together solely on the basis of microscopic histological features may be inappropriate even if they share a number of vascular risk factors. Whereas in systemic amyloidosis it is unequivocally clear that the amyloid deposits cause tissue damage and organ dysfunction leading to clinical disease, the role, effects, and significance of the microscopic amyloid deposits in Alzheimer disease and (DM-2) are unknown and the subject of much speculation. The presence of SAP in the microfibrils of elastic membranes throughout the body has been known for long time, thus providing a plausible answer for its presence in plaque.

Data from animal and human studies suggest that circulating and/or local fibrocytes contribute to chronic inflammatory condition. It has been shown that fibrocyte
differentiation is suppressed by SAP \(^{128}\). This may be important in chronic diseases such as periodontitis \(^{129}\). The effect of periodontal treatment on SAP levels is however unclear. While one study reported elevated GCF levels of SAP after treatment \(^{112}\), another study reported reduced serum levels following conventional periodontal therapy \(^{130}\).

**Serum amyloid A (SAA)**

SAA is a highly conserved, acute-phase protein synthesized predominantly by the liver. After secretion into the circulation, it associates with high-density lipoprotein (HDL) particles. During acute inflammation, SAA levels may rise up to 1000-fold, and under these conditions, SAA displaces apolipoprotein A-I from HDL, thus becoming the major apolipoprotein of circulating HDL3. SAA exhibits significant immunological activity by, for example, inducing the synthesis of several cytokines and by being chemotactic for neutrophils and mast cells. It exerts many of its immunological activities by binding and activating cell-surface receptors, including Toll-like receptor (TLR) 2 and 4. SAA recently has been shown to activate the inflammasome cascade, which has a role in immune activation, thus further stressing its role of SAA in immunomodulation \(^{131}\).

**Clinical importance of SAA**

SAA has been considered to have a key role in the pathogenesis of amyloid A-type amyloidosi\(\text{s}\), but current knowledge supports the fact that it participates as well in the pathogenesis of chronic inflammatory diseases, such as rheumatoid arthritis and atherosclerosis \(^{131}\). Full-mouth tooth extraction in advanced periodontitis patients significantly reduced plasma level of SAA \(^{132}\). Untreated periodontitis, together with excessive deposition of amyloid fibrils derived from SAA protein, causes systemic amyloidosis, a serious inflammatory disorder \(^{133}\).

The clinical effect of periodontal therapy on SAA remains to be clarified \(^{40}\). A clinical study of our group investigated the effects of photodynamic therapy as adjunct to ultrasonic debridement in residual periodontal pockets on GCF biomarkers. A significant decrease in the level of SAA and CRP, fibrinogen, procalcitonin, and α2M was observed six months after treatment \(^{134}\).
Ferritin

Ferritin is an intracellular protein that stores iron and releases it in a controlled manner. The amount of ferritin stored reflects the amount of iron stored. The protein is produced by almost all living organisms, including algae, bacteria, higher plants, and animals. In humans, it acts as a buffer against iron deficiency and iron overload. Ferritin concentrations increase in the presence of an infection or a malignant tumor; this is believed to reflect the effort of the host to counter the infective agent's attempt to bind iron from the host's tissue. The inflammatory response may cause ferritin to migrate from the interstitial space to plasma, elevating the serum ferritin level, in order to deny iron to the infective agent.

Clinical importance of ferritin

A normal ferritin blood level stays between 30–300 ng/ml for males, and 15–200 ng/ml for females. If the ferritin level is low, there is a risk for lack of iron, which could lead to anemia. To verify this, low serum ferritin is the most specific lab test for iron deficiency anemia. However it is less sensitive, since its levels are increased in the blood by infection or any type of chronic inflammation. In adolescents and teenagers, ferritin levels that are low but yet above those causing anemia and sickness (12 to 50 ng/ml) may cause symptoms of restless legs syndrome. Vegetarianism may contribute to low levels of serum ferritin, according to a study that found 40% of vegetarians tested with low serum ferritin levels. On the other hand, ferritin is also an acute-phase reactant, and hence elevated in the course of disease. A normal C-reactive protein level can be used to exclude elevated ferritin caused by acute phase reactions. According to a study on anorexia nervosa patients, ferritin can be elevated during periods of acute malnourishment, perhaps due to iron going into storage as the intravascular volume and thus the number of red blood cells falls. A recent report demonstrated that ferritin is predominantly expressed in periodontal ligament (PDL) tissues and positively regulates the cytodifferentiation and mineralization of PDL cells. Since these cells are the principal cells for periodontal wound healing, that finding suggest that ferritin is involved in the homeostasis, remodeling and regeneration of periodontal tissues.

In a clinical study conducted on 19 female subjects diagnosed with iron deficiency anemia and periodontitis, GCF and serum levels of Ferritin were evaluated 3 months after SRP. The GCF ferritin concentration significantly decreased, however, no
significant correlation was found between serum and GCF ferritin levels 144.

**Fibrinogen**

Fibrinogen is a plasma glycoprotein synthetized by the liver. The conversion of fibrinogen to fibrin is catalyzed by thrombin and plays a key role in clot formation and stabilization. Moreover, fibrinogen induces platelet activation and aggregation by binding to the platelet fibrinogen receptor glycoprotein GPIIb/IIIa 145. It is catabolized through normal protein degradation, the coagulation process, and other unknown pathways 115.

**Clinical importance of fibrinogen**

Normal serum fibrinogen concentrations are below 4 mg/ml while higher concentrations have been associated with severe pathological conditions such as squamous cell carcinoma 146. Fibrinogen deficiency is associated with uncontrolled bleeding and compromised survival. Thus, regulation of fibrinogen availability is critical to survival in trauma patients 147. On the other hand, several animal and human studies showed a specific link between fibrinogen and the progressive and metastatic behavior of tumor cell 148 149 150 151.

A growing body of evidence from epidemiological studies implicates moderate and severe periodontitis with cardiovascular disease (CVD), especially coronary heart disease (CHD), and suggests that periodontitis may be one of the risk factors for CHD. But the mechanisms underlying the association between periodontitis and CVD are still not clear. Fibrinogen, can be increased by approximately 2- to 10-fold during acute-phase response. A gene polymorphism of G/A variability in the -455 locus of the β-fibrinogen promoter region has been shown to be associated with elevated plasma fibrinogen levels. It is now widely accepted that plasma fibrinogen levels are strongly associated with an increased risk of cardiovascular disease CVD and may be an independent risk factor of CHD 152.

Periodontal inflammation can cause local production of pro-inflammatory cytokines that may exert an effect on the hepatic synthesis of fibrinogen and the elevation of fibrinogen plasma and serum levels, which may in turn lead to increased inflammatory activity in atherosclerotic lesions and accelerated development of CHD. Therefore, fibrinogen may represent a possible candidate molecule that links periodontal disease with CHD. Still, more research is needed on this association 120.
It has been shown that a higher percentage of chronic periodontitis patients exhibit genotypes associated with higher plasma fibrinogen levels than healthy individuals. In addition, periodontitis patients have significantly higher fibrinogen levels compared to healthy individuals.\textsuperscript{153}

There is limited evidence on the effect of periodontal therapy on fibrinogen levels.\textsuperscript{40} Adjunctive photodynamic therapy to ultrasonic debridement showed the ability to reduce GCF fibrinogen levels.\textsuperscript{134}

**Tissue plasminogen activator (t-PA)**

Plasminogen activators are serine proteases that form part of the complex enzyme cascade involved in fibrinolysis. These enzymes convert plasminogen into plasmin, a trypsin-like serine protease, that is not only responsible for the degradation of fibrin, but also contributes directly and indirectly, via conversion of latent collagenase into active collagenase, and to the degradation and turnover of the extracellular matrix.\textsuperscript{154}

Plasmin can be formed locally at sites of inflammation by limited proteolysis of its inactive precursor, plasminogen, which circulates in plasma and interstitial fluids.\textsuperscript{155} Either urokinase-type plasminogen activator (u-PA) or tissue type plasminogen activator (t-PA) activates plasminogen. These catalytic reactions generally take place at the plasma membrane (u-PA) or on a fibrin surface (t-PA). A wide range of mesenchymal, epithelial and endothelial cells produces these activating enzymes in response to a variety of cytokines and growth factors. Therefore, at sites of inflammation, the chance for up-regulation of the plasminogen activation system is high.

The resultant activated plasmin can degrade several substrates including extracellular matrix macromolecules (excluding collagens) and fibrin. The activities of plasmin and its activating proteinases are regulated extracellularly through a number of proteinase inhibitors including α2-macroglobulin, α1-proteinase inhibitor, α2-antiplasmin, plasminogen activator inhibitor-1 and 2 (PAI-1, PAI-2).\textsuperscript{156}

**Clinical importance of t-PA**

In health, serum levels of t-PA have been reported within a range of 0.1 to 12 ng/ml.\textsuperscript{157} The plasminogen activating system has been of interest for studies of tumor growth and spread as well as destructive inflammatory diseases. Destructive inflammatory diseases are found in various organs and tissues, such as lungs, joints and tooth supporting tissues. These conditions have in common the breakdown of connective tissue components through proteolytic activity.\textsuperscript{159} In clinical studies on periodontitis it
has been shown that t-PA and PAI-2 may play a significant role in periodontal tissue destruction and tissue remodeling. t-PA and PAI-2 in GCF may serve as surrogate marker for presence of the periodontal diseases and may be used to assess biological reaction to therapy\textsuperscript{160}.

Recently, a study intended to evaluate GCF levels of t-PA and PAI-2 in both aggressive and chronic periodontitis before and after non-surgical periodontal treatment. Significant reduction of PAI-2 was found only in chronic periodontitis patients\textsuperscript{161}. These results were opposite to a previously mentioned study that found GCF levels significantly elevated after the therapy\textsuperscript{109}.

**Procalcitonin (PCT)**

PCT is a protein produced by the C cells of the thyroid gland as a precursor protein of calcitonin. The origin of the synthesis and secretion of PCT during infections are considered to be extra-thyroidal. Its production was demonstrated in the lung, liver, pancreas, colon, and other organs. PCT behaves like an acute phase protein such as CRP, and therefore the production increases as a result of inflammatory stimulation, including infection\textsuperscript{162}.

**Clinical significance of PCT**

The blood levels of PCT may rise to 100 ng/ml in severe infectious diseases, whereas the normal value is approximately 1 ng/ml\textsuperscript{163}. Measuring the level of PCT can be used as a marker for severe sepsis caused by bacteria\textsuperscript{164}. PCT has the greatest sensitivity (85\%) and specificity (91\%) for differentiating patients with systemic inflammatory response syndrome from those with sepsis, when compared with IL-2, IL-6, IL-8, CRP and TNF-α\textsuperscript{165}. For the diagnosis of bacteremia, PCT had a sensitivity of 76\% and specificity of 70\%\textsuperscript{166}. Evidence is emerging that PCT levels can reduce unnecessary antibiotic prescribing to people with lower respiratory tract infections\textsuperscript{167}.

Patients diagnosed with severe periodontitis showed higher levels of salivary PCT than did those with moderate periodontitis or periodontally healthy subjects\textsuperscript{168}. Once again, a clear effect of periodontal therapy on PCT is not yet established as GCF levels were reported to decrease or to increase following therapy\textsuperscript{109,130}. 
Serological analysis of inflammatory markers in periodontology

Measuring and evaluating soluble cytokines and other analytes in serum and plasma has become important in the management of many diseases. There is an increasing demand for rapid, precise and cost-effective methods for the assessment of biological markers in clinical practice and for clinical research.

The two classical methods for the assessment of most biological markers are Western blotting and Enzyme-linked immunosorbent assay (ELISA). In the former assay, proteins are resolved by size on a protein gel, transferred to a membrane and probed with an antibody to the protein of interest. In ELISA, the well of a microtiter plate is coated with antibodies to capture the protein of interest. The protein sample is placed in that well, a secondary antibody is added to the same antigen and probed for that secondary antibody.

Both assays depend on immune-recognition of specific antigens and therefore, both are well-accepted tests. The disadvantage is that both can report on only one antigen at a time ("single-plex"). For the simultaneous study of multiple analytes a series of Westerns or ELISAs have to be performed, which requires a large sample volume and is cost- and time-intensive.

Unlike single-target assays, multiplex assays enable researchers to measure multiple analytes simultaneously, anywhere from two to dozens at once. Multiplex assays exist in two basic forms: Planar and bead-based. The former is essentially a direct extension of the ELISA while the latter is a solution-based alternative. In the next section we will focus our review on the multiplex-bead assay Bio-Plex® since it is the one used for the current research project, and we will compare it to the classic alternative ELISA. Figure 2 shows the Bio-Plex 200 system at the laboratory of the Division of Periodontology and Oral Pathophysiology, School of Dental Medicine, University of Geneva.
Multiplex Bead Array Assay (MBAA) using xMAP technology

By multiplexing different assays, the Bio-Plex® suspension array system is able to perform up to 100 tests from only a single sample. This is mainly due to the ability of this technology to use 100 distinct colored beads. This distinction is a result of the use of two fluorescent dyes at distinct ratios.$^{169}$

In a first step comes when one antibody to a specific analyte is attached to a set of beads with the same color. A second antibody is then used to quantify the bound antigen. Following incubation of the capture antibody-coupled beads with antigen standards or samples for a specific time, the plate is washed to remove unbound materials. Next, it is incubated with biotinylated detection antibodies. Then the unbound biotinylated antibodies are washed away and the beads are incubated with a reporter streptavidin-phycoerythrin conjugate (SA-PE). The excess SA-PE is then removed and the beads are passed through the array reader, which measures the fluorescence of the bound SA-PE. The method used in our laboratory for washing out antibodies and excess SA-PE is by vacuum filtration.

During laser excitation detection the contents of each microplate well are drawn into the array reader and precision fluidics align the beads in single file through a flow cell,
where two lasers excite the beads individually. The first laser excites the dyes in each bead, identifying its spectral address (Figure 3, top). The second laser excites the molecule associated with the bead, which allows quantitation of the captured analyte. Digital signal processors record the fluorescent signals for each bead and translate the signals into data for each bead-based assay (Figure 3, bottom).
Figure 3

Beads are pushed through a detection chamber. The reporter laser (532 nm) interrogates fluorescent reporter to measure analyte concentration. The classification laser (635 nm) interrogates internal dye to identify bead regions.
MBAA and ELISA

MBAA offers several advantages over the classical methods like ELISA. The major technical difference is that captured antibodies in MBAA are attached to the bead surface in a covalent manner. This specific link permits a greater surface area to react with the analyte. Other differences between the two methods are mainly in term of sample volume, cost and effort. A side-by-side comparison of analyzing 27 cytokines in 80 samples by ELISA or Bio-Plex is made in Table 1.

Table 1
Side by side comparison of analyzing 27 cytokines in 80 samples by ELISA or Bio-Plex. Adapted from Houser 169.

<table>
<thead>
<tr>
<th></th>
<th>ELISA</th>
<th>Bio-Plex</th>
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<tr>
<td><strong>Number of cytokines</strong></td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td><strong>Number of samples</strong></td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td><strong>Total data points</strong></td>
<td>2160</td>
<td>2160</td>
</tr>
<tr>
<td><strong>Number of 96-well plates</strong></td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td><strong>Data points per plate</strong></td>
<td>80</td>
<td>2160</td>
</tr>
<tr>
<td><strong>Total time required</strong></td>
<td>&gt; 60 hours</td>
<td>3 hours</td>
</tr>
<tr>
<td><strong>Sample volume</strong></td>
<td>&gt;1ml</td>
<td>12.5 µl</td>
</tr>
<tr>
<td><strong>Assay range (dynamic range)</strong></td>
<td>3-4 logs</td>
<td>5-6 logs</td>
</tr>
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</table>

According to Elshal and McCoy 170 several studies have demonstrated good correlations, but often-poor concurrence of quantitative values, between MBAA and corresponding ELISA measurements. It has been shown that a comparison of randomly selected bead array assays and ELISAs will likely demonstrate substantial differences. However, if
comparisons are made between both assays, which use identical capture and reporter antibodies as well as similar diluents and serum blockers, variability will be minimized, correlations will be good, and similar quantitative values may be achieved. Given this, any study that involves sequential monitoring of patients, or other samples, should be performed using only a single technique, one platform, and one commercial vendor for all samples. As illustrated by Khan et al. 171, although multiplex assays from different vendors will show similar trends in cytokine levels, the absolute levels of cytokines measured will vary. Therefore, even MBAA assays from different vendors should not be considered interchangeable. When transitioning from an existing assay to a new MBAA assay, it might be possible to establish the systematic bias between the two assays and at least establish some basis for comparing data.

Periodontal therapy and systemic diseases

Evidence in human subjects demonstrating the beneficial effects of periodontal therapy on cardiovascular disease outcomes is limited. A study by D’Aiuto et al.39 showed that periodontitis patients when treated with SRP exhibited significant serum reducing in cardiovascular disease markers, CRP, and IL-6. Patients who clinically responded to periodontal therapy in terms of pocket depth reductions where four times more likely to exhibit a decrease of CRP in serum relative to patients with poor clinical periodontal response. A recent clinical trial tried to evaluate the effect of periodontal therapy in reducing systemic inflammation in patients with metabolic syndrome (MetS) and reduces cardiovascular risk. The primary and secondary outcomes were changes in serum CRP and fibrinogen levels, respectively. Clinical parameters were assessed at baseline and every three months until 12 months after therapy. After 9 months, a significant reduction of clinical inflammation was achieved with and without adjunctive systemic antibiotics, and a significant reduction of CRP levels was obtained in patients with MetS 172. More reduction of fibrinogen was observed up to 12 months in the adjunctive antibiotic group.

Periodontitis has effects that go beyond the oral cavity and its treatment and prevention may contribute to the prevention of atherosclerosis. According to a recent statement of the American Heart Association, available data indicate a general trend toward a periodontal treatment-induced suppression of systemic inflammation and improvement of non-invasive markers of atherosclerotic vascular disease and
endothelial function. The effects of periodontal therapy on specific inflammatory markers are however not consistent across studies, and their sustainability over time has not been established convincingly. Determinants of variability in these responses remain poorly understood. In addition, transient proinflammation and deranged endothelial functions are observed after intensive therapy for PD 173.

During pregnancy, there are profound perturbations in innate and adaptive immunity that have an impact on the clinical course of a number of infectious diseases, including those affecting periodontal tissues. Pregnancy-associated increases in gingival inflammation are a well-documented phenomenon that is universally accepted by the scientific community 174. Plaque induced periodontitis increases in clinical extent and severity during the course of a normal pregnancy. A positive association between GCF mediators, such as interleukin-1β, prostaglandin E2, and TNF-α, and adverse pregnancy outcomes has been documented 175. Periodontal therapy provided during pregnancy successfully reduced periodontal inflammation and GCF levels but did not have a significant impact on serum biomarkers 176.

The clinical beneficial effects of periodontal therapy during pregnancy can be summarized from two systematic reviews. Following analysis of 13 randomized clinical trials Chambrone, et al. 177 concluded that maternal periodontal disease treatment did not decrease the risk of preterm birth and/or low birth weight. In another recent review 178 a meta-analysis was done on twelve randomized trials. The pooled estimates showed no significant reduction of preterm birth with periodontal treatment and the author concluded that there is an important heterogeneity between randomized trials that evaluated this clinical effect.

The results from recent studies using animal models indicate that periodontitis does influence the progression of DM. Thus, treatment of periodontitis to reduce low-grade systemic chronic inflammation that exists in DM subjects may help glycemic control and help reduce the extent of organ damage 179. A systematic review on clinical trials concluded that poorly controlled diabetes might be considered a risk factor for increased severity of periodontitis 180. Meta analysis revealed that there is some evidence of improvement in metabolic control in people with DM, following periodontal treatment. There are few studies available, and individually these lacked the power to detect a significant effect. Most of the participants in these studies had poorly controlled Type 2 DM with little data from randomized trials on the effects on people with Type 1 DM 181. A recent clinical trial evaluated the effects of full-mouth and partial-mouth SRP
on clinical parameters and local levels of cytokines including TNF-α, IFN-γ, IL-17, IL-23, IL-4, and osteoclastogenesis-related factors in type 2 DM subjects with chronic periodontitis. Both methods yielded benefits in clinical parameters and resulted in a similar modulation of cytokines and osteoclastogenesis-related factors at 12 months in type 2 DM subjects.

Antibiotic therapy and periodontal treatment

Systemic vs. local antibiotics

Antibiotics, which are also known as antibacterial, can be described as medications that destroy or slow down the growth of bacteria. In dentistry, there are two conventional routes for administering antibiotics: systemic and local. A major advantage of the local route is that the concentration of the drug is higher than if it is administered systemically. Local antibiotics require that the infection be confined to the site of the disease such as residual defects. However, oral bacteria and periopathogens in particular, can be found in other oral sites like the dorsum of the tongue and tonsillar crypts and therefore, has limited benefits. On the other hand, systemic adverse events and serious drug reactions are a greater risk for the systemic administration, (Table 2).

Table 2. Comparison of local and systemic antimicrobial therapy

<table>
<thead>
<tr>
<th></th>
<th>Systemic administration</th>
<th>Local administration</th>
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<tbody>
<tr>
<td>Drug distribution</td>
<td>Wide</td>
<td>Narrow effective range</td>
</tr>
<tr>
<td>Drug concentration</td>
<td>Variable levels in different body compartments</td>
<td>High dose at treated sites, low levels else where</td>
</tr>
<tr>
<td>Therapeutic potential</td>
<td>May reach widely distributed microorganisms better</td>
<td>May act locally on biofilm-associated bacterial better</td>
</tr>
<tr>
<td>Problems</td>
<td>Systemic side effects</td>
<td>Reinfection from non-treated sites</td>
</tr>
<tr>
<td>Clinical limitation</td>
<td>Requires good patient compliance</td>
<td>Requires infection to be limited to the treated site</td>
</tr>
</tbody>
</table>
Methods to deliver antimicrobial agents into periodontal defects include pocket irrigation, placement of drug-containing ointments and gels, and devices for sustained drug release. Firm conclusions about the efficacy of these local antimicrobial therapies could not be reached in two systemic reviews. When compared to the systemic route, local antibiotics were shown less cost-effect than systemically delivered antimicrobials.

Several systemic antibiotics have been tested in clinical trials, either alone or combined with another type of treatment. These include amoxicillin (with or without calvulanic acid), azithromycin, clindamycin, doxycycline, metronidazole, spiramycin, and tetracycline. Amoxicillin has been the preferred antibiotic in the treatment of periodontitis due to its activity against periodontal pathogens at levels reached in GCF. Nitroimidazoles (metronidazole and ornidazole) inhibit DNA synthesis. Metronidazole acts specifically against the obligately anaerobic microbiota, which includes many but not all suspected periodontal pathogens.

**Amoxicillin plus metronidazole**

Some combined antibiotics may offer an enlarged spectrum of activity and may induce less bacterial resistance than the single agents. Combination therapy may be especially useful when acting against a complex subgingival microbiota that might harbor multiple pathogenic species with different antimicrobial susceptibility. A synergistic effect against *Aggregatibacter actinomycetemcomitans* has been noted in vitro between metronidazole and its hydroxyl metabolite as well as between the two compounds and amoxicillin.

A recent systematic review concluded that systemic antimicrobial therapy using a combination of amoxicillin and metronidazole as an adjunct to SRP can enhance the clinical benefits of non-surgical periodontal therapy with the full-mouth weighted mean change for clinical attachment level showed a gain of 0.94 mm in favor for this regime. Another systematic review in 2008 tried to assess the benefits of prescribing antibiotics either during the non-surgical or the surgical phase of therapy remained inconclusive.

In 51 patients diagnosed with chronic periodontitis, systemic metronidazole plus amoxicillin significantly improved the 6-month clinical outcomes of extensive full-mouth non-surgical periodontal debridement provided in 48 hours, thereby significantly reducing the need for additional therapy. Another study demonstrated the adjunctive benefits of the same antibiotic regime after full mouth debridement in 41
patients with generalized aggressive periodontitis. In summary, the data from these trials explain the strength and consistency of the beneficial effect of adjunctive amoxicillin and metronidazole, independent of the diagnosis and varied treatment protocols.

We showed recently that the clinical benefit from amoxicillin plus metronidazole was independent of the presence or absence of specific periodontal marker organisms, i.e., *A. actinomycetemcomitans*. This is in contrast to the notion that it may be advantageous to specifically eliminate target bacteria only and to allow therefore, the growth of potential beneficial microorganisms. That was the basis for an argument to propagate narrow-spectrum antibiotics for periodontal treatment like metronidazole alone. However, in two comparative studies, better clinical outcomes were reported if patients were treated with metronidazole plus amoxicillin than if metronidazole alone was administered.

**Adverse drug reactions of adjunctive amoxicillin plus metronidazole to periodontal therapy**

Like all medicines, antibiotics have the potential to cause adverse drug reactions. The common side effects of almost all antibiotics are stomach problems such as diarrhea, nausea and vomiting. These side effects happen because antibiotics may lead to inflammation of the intestine lining, and disruption of the species composition in the intestinal flora, and overgrowth of pathogenic bacteria. The intestines therefore will be less able to absorb water and nutrients from food, resulting in diarrhea. Penicillin type antibiotics such as amoxicillin can cause allergic or hypersensitivity reactions such as hives (rash, itch, flushing, fever), fever and breathing problems. In rare situations, a person may experience a severe or immediate allergic reaction to the antibiotic (anaphylaxis). Common adverse drug reactions of systemic metronidazole therapy include nausea, diarrhea, and/or metallic taste in the mouth. Infrequent adverse events include hypersensitivity reactions. Alcohol consumption enhances these symptoms, because imidazoles affect the activity of liver enzymes. Therefore, careful patient orientation is necessary before starting the drug administration. Peripheral neuropathies, characterized by numbness or paraesthesia of an extremity, have been reported from isolated cases. Therefore, metronidazole should be discontinued immediately if abnormal neurological signs appear. Metronidazole has shown evidence of carcinogenic activity in mice and rat studies following chronic oral administration, but not in other tested species. Due to inadequate evidence, metronidazole is not considered as a risk factor for cancer in humans.
According to Mombelli 2012, the frequency and consequences of adverse effects of antibiotics have to be balanced against potential health consequences of not rapidly suppressing a periodontal infection, and with the inconvenience, discomfort and financial consequences of further therapy. The conventional approach sometimes expands treatment over several months, while SRP plus metronidazole and amoxicillin may be able to resolve the infection within a few days. This combination therapy has been shown to decrease the clinical signs of inflammation and inflammatory biomarkers in GCF more profoundly than SRP alone.  Although not directly confirmed so far by a clinical trial, it seems preferable, from a general health point of view, to let patients benefit early from the positive systemic effects of successful periodontal therapy.


Aim of the study

Currently available data on periodontal systemic associations are rather heterogeneous and the evidence that periodontal therapy has an impact on systemic health is limited. Technological developments have made it possible to detect and quantify a range of various biological markers in relatively small fluid specimens simultaneously. The quantification of multiple markers in one sample can provide information on interactions that are inaccessible by studying single markers individually. Studies on changes of serological inflammatory markers after periodontal therapy conducted so far focused on mean or median changes of single serological markers. The presence, and the disappearance, of multiple extreme values may, however, be biologically important in affected individuals, even if this does not concern all participants of a trial.

The aims of this study were:

1. Assessing the response to non-surgical periodontal therapy by measuring a range of 24 analytes in serum of periodontally diseased patients before and three months after therapy, with a focus on subjects with high levels of multiple biomarkers before therapy.

2. Elucidating whether adjunctive systemic antibiotic treatment will influence the systemic response to therapy.

3. Evaluating the adverse events related to the given therapeutic regime and put them into perspective with potential systemic benefits of more profoundly suppressing the inflammatory status.
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