DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
124

PERI-IMPLANT DISEASE: PATHOGENESIS, DIAGNOSIS AND TREATMENT IN VIEW OF BOTH INFLAMMATION AND OXIDATIVE STRESS PROFILING

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Dissertation is accepted for the commencement of the degree of Doctor of Medical Sciences on April 19th, 2006 by the Council of the Faculty of Medicine, University of Tartu, Estonia

Commencement: June 14th, 2006

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Publication of this dissertation is granted by University of Tartu

ISSN 1024–395X
ISBN 9949–11–358–X (trükis)
ISBN 9949–11–359–8 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr. 287

To my son Simon

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II. S.Liskmann, T.Vihalemm, O.Salum, K.Zilmer, K.Fischer, M.Zilmer. Correlations between clinical parameters and Interleukin-6 and Interleukin-10 levels in saliva from totally edentulous patients with peri-implant disease. In press: *The International Journal of Oral & Maxillofacial Implants*

III. S.Liskmann, T.Vihalemm, O.Salum, K.Zilmer, K.Fischer, M.Zilmer. Characterization of the antioxidant profile of human saliva in peri-implant health and disease. In press: *Clinical Oral Implants Research.* Publication is anticipated for Vol.18, 2007

IV. T.Vihalemm, S.Liskmann, U.Kokassaar, M.Zilmer. Multicomponental mixture of antioxidants and patients with periodontitis. Free Radical Research 2002; **36**: 120–121

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ABBREVIATIONS

ABTS	2,2’-azino-bis-[ethylbenzothiazoline sulfonate]
APC	antigen-presenting cell
AST	aspartate aminotransferase
ATP	adenosine triphosphate
BOP	bleeding on probing
CAT	catalase
CI	confidence interval
DNA	deoxyribonucleic acid
IL	interleukin
INF	interferone
GAG	glycosaminoglycan
GCF	gingival crevicular fluid
GI	gingival index
GSH	glutathione, reduced
GSSG	glutathione, oxidized
LA	linolenic acid
LOOH	lipid hydroperoxides
MMP	matrix metalloproteinase
MPO	myeloperoxidase
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
OxS	oxidative stress
PISF	peri-implant sulcus fluid
PGE	prostaglandin E
PMN	polymorphunuclear lymphocytes
PPD	probing pocket depth
ROS	reactive oxygen species
SD	standard deviation
SOD	superoxide dismutase
TAP	total antioxidative protection
TAS	total antioxidative status
TBARS	thiobarbituric acid reactive substances
TNF	tumor necrosis factor

1. INTRODUCTION

Today, osseointegration is a routine method in the rehabilitation of partial or total edentulism. For the last 20 years, favorable long-term results of tissue-integrated prostheses have been reported in completely and partially edentulous patients (Adell et al. 1981, Albrektsson et al. 1988, Jemt & Lekholm 1993, Nevins & Langer 1993, Buser et al. 1997, Hultin et al. 2000). Despite the predicable treatment results, with most failures occurring during initial healing and the first year of loading, complications do arise during maintenance and retention of implants. The tissues supporting osseointegrated dental implants are susceptible to disease that may lead to implant loss (Malmström et al. 1990, Fardal et al. 1999). Peri-implant disease is an inflammatory reaction affecting the tissues surrounding dental implants. While peri-implant mucositis is a reversible inflammation of the soft tissues surrounding dental implant, peri-implantitis is defined as an inflammatory reaction affecting the tissues surrounding osseointegrated dental implants resulting in loss of supporting bone (Albrektsson & Isidor 1994, Tonetti & Schmidt 1994, Mombelli & Lang 1998, Esposito et al. 1999). Peri-implantitis has also been described as "a site-specific infection yielding many features in common with chronic adult periodontitis" (Mombelli et al. 1987). Peri-implantitis has been meta-analysed to account for 10–50% of implant failures after the first year of loading (Esposito et al. 1998a, 1998b).

The view that microorganisms play a major role in the development of peri-implant disease is supported by several clinical findings. A cause-related effect between plaque accumulation and peri-implant mucositis has been shown in animals (Berglundh et al. 1992) and in humans (Pontoriero et al. 1994). The microbial colonization of implants follows the same pattern as that around teeth (Leonhard et al. 1992, Lang et al. 1993). A positive correlation has been found between plaque accumulation and marginal bone loss around oral implants (Schou et al. 1992, Lindquist et al. 1996). In sites with peri-implant disease a complex microbiota is established, closely resembling that found in adult periodontitis (Rams & Link 1983, Becker et al. 1990, Rams et al. 1991, Rosenberg et al. 1991, Alcoforado et al. 1991, Aughthun & Conrads 1997). It is clear that the role of microflora in peri-implant disease is in the initiation of the inflammatory response of the host organism. This inflammatory host response is tightly associated with the neutrophil activation and subsequent development of high-grade oxidative stress (OxS).

Neutrophils (PMN) are within the first line of host defence, and, by their ability to phagocytize microbes, they can protect the host from infection.

However, in addition to the direct involvement of neutrophils in the defense against invading pathogens, the neutrophils' role in mediating tissue destruction in inflammatory diseases is also a significant parameter in pathogenesis (Weiss 1989, Baumann & Gauldie 1994). Neutrophil-mediated tissue injury plays an

important role in the pathogenesis of several significant diseases (Van Dyke & Serhan 2003).

Until recently, defects associated with neutrophil functions were believed to predispose individual to infection. However, there is a growing body of evidence suggesting that the neutrophil abnormalities in periodontal disease are the result of a chronic hyperactivated or "primed" state of the periodontitis neutrophil. Under this new paradigm, the neutrophil is not hypofunctional or deficient, but hyperfunctional, and it is the excess activity (accompanied also by rapid potent production of reactive species) and release of toxic products from the cell that are responsible, in part, for the tissue destruction in chronic periodontal inflammation (Van Dyke & Serhan 2002).

Reactive oxygen species or ROS (including oxygen free radicals) produced by PMNs can attack every biologically relevant molecule, including proteins, lipids, carbohydrate, and nucleic acids (Badwey & Karnovsky 1980). *In vitro* studies have shown that ROS are capable of degrading a number of extracellular matrix components including proteoglycans leading to a loss of structural integrity of the periodontal tissues (Waddington et al. 2000). Principal ROS are superoxide anion (O_2^-), hydroxyl radicals and hydrogen peroxide. In addition to their direct oxidative attack, ROS can also modulate various cellular activities that are important mediators in the sequence of events leading to tissue injury (Gasic et al. 1991).

Neutrophil-mediated tissue damage in the periodontium was first demonstrated by Deguchi et al (1990). Similar findings were reported for neutrophil and gingival epithelial cell interactions (Altman et al. 1992), where it was demonstrated that human neutrophils could lyse epithelial cells in an *in vitro* model mediated by myeloperoxidase-hydrogen peroxide interactions. In addition to the direct deleterious activities of neutrophils on host periodontal tissues, superoxide anion generation was shown to be elevated in both resting and stimulated aggressive periodontitis neutrophils (Van Dyke et al. 1986).

In summary, hyperfunctional neutrofils produce excessive amounts of ROS that results in high-grade OxS. It should be emphasized that any profound inflammation is linked to high-grade OxS (Stocker & Keaney 2004).

Peri-implant disease may have a multifactorial background where an aberrant host response in conjunction with the bacterial challenge may contribute to the development of tissue destruction around implants. Clarification of factors of importance for peri-implant disease may make it easier to predict which patient or implant is at risk for peri-implant complications during maintenance and retention of implants. The aim of the present study was therefore to characterize host response to microbial challenge by testing pro- and anti-inflammatory markers in patients with signs of peri-implant disease and describe OxS profile of this disease. Thus, the purpose of the study was to profile peri-implant disease in view of both inflammation and OxS.

The first part of this study was performed to assess whether an antioxidant-strategy may have beneficial impact in systemic treatment of patients with soft and hard tissue destruction, i.e. periodontitis.

The second part of the study investigated the correlation of myeloperoxidase (MPO) levels in peri-implant sulcus fluid (PISF) and peri-implant health and disease in partially edentulous patients.

The third part of the study compared the levels of pro-inflammatory (IL-6) and anti-inflammatory (IL-10) markers in saliva in patients with peri-implant disease and healthy controls. Both these groups included edentulous patients.

Finally, the fourth part of this study was aimed to characterize the anti-oxidant profile of saliva in patients with peri-implant disease and healthy controls. These groups also included edentulous patients.

2. REVIEW OF THE LITERATURE

2.1. Peri-implant disease: peri-implant mucositis and peri-implantitis

Definition

At the First European Workshop on Periodontology, peri-implantitis was defined as an inflammatory process affecting the tissues around an osseointegrated dental implant in function, resulting in loss of supporting bone. Peri-implant mucositis was defined as reversible inflammatory changes of the peri-implant soft tissues without any bone loss (Albrektsson & Isidor 1994). Both these pathologic conditions can be placed in the general category of peri-implant disease (Lang & Karring 1994).

Peri-implantitis begins at the coronal portion of the implant, while the most apical portion of the implant maintains an osseointegrated status (Sanz et al. 1990, Lindhe et al. 1992, Lang et al. 1993). This means that implant is not clinically mobile until the late stages, when bone loss has progressed to involve the complete implant surface (James 1973, Newman & Flemming 1988). Recent studies introduced the term retrograde peri-implantitis as a lesion around the most apical part of osseointegrated implant (Quirynen et al. 2005).

Incidence

The prevalence of peri-implant mucositis has been reported in the range of 8–44% (Adell et al. 1986, Lekholm et al. 1986a, Tolman & Laney 1992, Smedberg et al. 1993, van Steenberghe et al. 1993, Bengazi et al. 1996, Jepsen et al. 1996, Behneke et al. 1997, Lekholm et al. 1999), while frequency of peri-implantitis has been reported in the range of 1–19% (van Steenberghe et al. 1990, Weber et al. 1992, Smedberg et al. 1993, van Steenberghe et al. 1993, Lekholm et al. 1999, Berglundh et al. 2002). The wide ranges for the frequencies are attributed to the differences in defining the two entities (Roos-Jansaker et al. 2003).

Many long-term studies present an average of the marginal bone loss around dental implants, reporting a mean decrease of crestal bone 0.9 to 1.6 mm during the first year of function. After the first year, mean annual rates of bone loss were reported as 0.05 to 0.13 mm (Adell et al. 1981**,** Albrektsson et al. 1986, Buser et al. 1991).

Susceptibility for peri-implantitis versus periodontitis

There is some controversy in the literature regarding the question whether the patients with previous history of periodontal disease are more susceptible to peri-implant disease compared to subjects without such a history. Several studies showed that microflora present in oral cavity before implantation determines the composition of the newly established microflora on implants (Koka et al. 1993, Leonhardt et al. 1993, Kohavi et al. 1994, Mombelli et al. 1995). The bacteria colonizing implants in the edentulous patients originate primarily from the surfaces of adjacent soft tissues. In partially edentulous patients, the dental microflora appears to be an additional important source of bacteria (Mombelli 2002). A retrospective study indicated that edentulous patients who lost their teeth due to periodontitis had a poorer outcome of implant therapy than edentulous subjects without such a history (Cune & Putter 1996). Due to their apparent susceptibility to periodontitis and based on high probability of transmission of periodontal pathogens, partially edentulous patients with a history of periodontitis are considered at an elevated risk of developing peri-implantitis (Mombelli & Lang 1992, Mombelli 2002).

Several other publications, however, proved the opposite. Nevins and Langer (1995) published data on the survival rate of Brånemark system implants (with a machined surface) in a group of partially edentulous patients with a clinical diagnosis of recalcitrant periodontitis (defined as no positive response to routine periodontal therapy and continuing loss of periodontal support). In contrast to what could be expected, both the survival rate and the stability of peri-implant tissues were comparable to what is generally reported for that implant system. Comparable observations were made by van Steenberghe and co-workers (1999) and Quirynen and co-workers (2001). These data together with the observations in long-term clinical studies (Adell et al. 1981, 1986, van Steenberghe et al. 1990, Lindquist et al. 1997) indicated that some implant configurations and surfaces might be more resistant to loss of "attachment" than teeth (Quirynen et al. 2002). These observations are, however, in contrast to reports on implants with rougher surface. Ellegaard and co-workers (1997) followed Astra and ITI system implants inserted in periodontally compromised partially edentulous patients. Abaout 76–86% of implants remained free from radiographic bone loss ≥ 1.5 mm at 36 months. After 5 years of loading, 45% of the ITI implants displayed marginal bone loss of 1.5 mm or more even though all patients participated in a periodontal supportive care program. Comparable data were reported in a longitudinal multi-center study on the same implant type (Brocard et al. 2000). The comparison of these results with those obtained for a population at low risk for periodontitis and using the same implant system (Buser et al. 1997), suggests that some implant types inserted in patients prone to periodontitis may pose an increased risk for marginal soft and hard tissue problems (Quirynen et al. 2002).

Etiology and pathogenesis

Microbial colonization

Biofilms form on all hard, non-shedding surfaces in fluid systems (Gristina, 1987). The oral cavity represents a fluid system in which microbiota present in saliva may colonize on teeth and artificial surfaces following the deposition of glycoprotein-containing pellicle. In addition, bacteria may accumulate in specific ecological niches that provide optimal ecological conditions for growth and division, such as periodontal pockets, tonsils and crypts, and folds of the tongue. Plaque formation on teeth has been described recently (Mombelli & Lang 1994). On oral implants, plaque formation has been studied using scanning electron microscopy and results showed that plaque formation patterns identified on implants are similar to those observed on teeth (Berglundh et al. 1992, Leonhardt et al. 1992, Ericsson et al. 1992, Abrahamsson et al. 1998).

Peri-implant mucositis

De novo plaque formation and its elicited host response have been studied histologically in a beagle dog model (Berglundh et al. 1992). It was found that the host response to the plaque formation on teeth and implants was similar.

Several investigatiors have studied the local defense mechanisms of the peri-implant soft tissue seal and compared them to those in dento-gingival unit. The production of inflammatory mediators and the expression of cytokines appear to be very similar in these two soft tissue types (Tonetti & Schmidt 1994). The experimental model of gingivitis, originally described by Löe et al. (1965), represens the ultimate proof for a cause-and-effect relationship between bacterial plaque accumulation and developing gingivitis. The same cause-and-effect relationship was demonstrated in peri-implant mucosa model as well (Pontoriero et al. 1994, Pontoriero et al. 1996).

Peri-implantitis

The marginal inflammatory tissue reactions in implants are similar to those encountered in gingivitis and periodontitis of teeth (Schou et al. 1992). Pathogenesis of gingivitis and periodontitis has been described recently (Kinane 2001). As soon as plaque accumulation begins, neutrophils are recruited to the periodontal pocket or the gingival crevice because of the chemotactic peptides, released by the bacteria. Furthermore, as bacteria damage the epithelial cells, they cause epithelial cells to release cytokines that further attract leucocytes (predominantly neutrophils) to the crevice. The neutrophils within the crevice can phagocytose and digest bacteria and therefore, remove these bacteria from the pocket. If the neutrophil becomes overloaded with bacteria, it degranulates. This causes tissue damage from toxic enzymes that are released from the neurophils. In conclusion, if there is an overload of microbial plaque, then the

neutrophils and the barrier of epithelial cells will not be sufficient to control the infection. In such instances, the gingival tissue will become inflamed and this is clinically seen as gingivitis. If inflammation extends from the marginal gingiva into the supporting periodontal tissues, this results in bone destruction and loss of attachment, a process termed periodontits.

The factors involved in bone destruction in periodontal disease are bacterial and host mediated. Bacterial plaque products induce the differentiation of bone progenitor cells into osteoclasts and stimulate gingival cells to release mediators that have the same effect (Hausmann et al. 1970, Schwartz et al. 1997). Several host factor released by inflammatory cells are capable to induce bone resorption *in vitro* and can play a role in periodontal disease. These include host produced prostaglandins and some cytokines (IL-1, TNF-α etc).

Plaque accumulation on the implant surface initiate the subepithelial connective tissue infiltration by large number of inflammatory cells and the epithelium appears ulcerated and loosely adherent. As the plaque front migrates apically, the clinical and radiographic signs of tissue destruction are seen around both implant and teeth; however, the size of the soft tissue inflammatory lesion and the bone loss is larger around implants (Lindhe et al. 1992, Leonhardt et al. 1992, Lang et al. 1993, Schou et al. 1993, 1996, Ericsson et al. 1995). In addition, the implant lesions extend into the supracrestal connective tissue and approximate/populate the bone marrow while the lesions associated with teeth do not. These studies suggest that plaque-associated soft tissue inflammation around implants may have more serious implications than marginal inflammation around teeth with a periodontal ligament.

Subgingival bacterial flora associated with clinically inflamed implant sites is different from that around "healthy" implants. These microbial shifts are comparable to those occurring around natural teeth, and the bacterial flora in chronic periodontitis and peri-implantitis have some similarities (Becker et al. 1980, Lekholm et al. 1986b, Jovanovic et al. 1988, Quirynen et al. 1991, Naert et al. 1992, Mombelli et al. 1995).

A subepithelial inflammatory response occurs and may play a role in continuing the inflammatory changes that cause the progressive breakdown (Koth et al. 1986, Sanz et al. 1990, Lang et al. 1993, Joanovic 1994). A marked difference has been documented between the bacterial morphotypes of the totally edentulous and the partially edentulous mouth (Mombelli et al. 1988, Apse et al. 1989, Philip et al. 1989, Quirynen & Listgarten 1990). The potential periodontal pathogens were shown to be less prevalent in the implant sulci of the totally edentulous mouth. This might indicate a higher susceptibility for peri-implantitis in the partially edentulous mouth (Mombelli & Lang 1992, Mombelli 2002).

It should be noted that the cause of peri-implant disease is multifactorial and there are many other factors that may contribute this process: biomechanical factors, traumatic surgical techniques, inadequate amount of host bone and

compromised host response (Quirynen et al. 1992, Jemt & Lie 1995, Jemt & Book 1996).

The excessive production of OxS-related reactive species is believed to be contributing factor in the pathogenesis of periodontitis (Battino et al. 2001). By our best knowledge there are no studies regarding the role of high-grade oxidative stress in the pathogenesis of peri-implant disease.

Diagnosis

The present methods for evaluating the supportive tissue status around dental implants have been based on radiographic and clinical parameters, such as gingival index (GI), probing depth, assessment of mobility and bleeding on probing (BOP) (Albrektsson et al. 1986). These parameters provide information about the extent of peri-implant tissue destruction, but do not reflect current tissue status nor can they predict the risk of peri-implant disease progression (Mombelli & Lang 1994). Radiographic bone loss becomes visible only after about 30% demineralization; evaluation of clinical parameters, including the GI, is often subjective. Although clinical and histological evidence exists that attachment levels around teeth and implants measured by probing are closely related to bone height (Quirynen et al. 1992, Ericsson & Lindhe 1993), probing pocket depth (PPD) and probing attachment level are inherently subject to examiner error and can be complicated by anatomic or abutment factors and prosthesis design (Fiorellini et al. 2000). Furtermore, the interpretation of the probing results around dental implants may be difficult due to the different tissue-to-implant interface. (Lang et al. 1994, Mombelli et al. 1997). As a result, the measurements of pocket depth may differ from those of the natural dentition, especially when inflammation is present. Clinically measurable implant mobility occurs only when osseointegration has failed. The use of an electronic device has been proposed (Periotest®, Siemens, Germany) to detect low degrees of mobility, however, the prognostic value of Periotest® reading has not been established (Teerlinck et al. 1991). Some recent studies show promising results using resonance frequency analysis in the assessment of implant stability (Lachmann et al. 2006), whereas other studies show that there are several factors (implant diameter etc) that influence the values for the resonance frequencies thus making quantitative assessment of implant stability difficult (Pattijn et al. 2006). BOP may not be an accurate indicator of peri-implant inflammation, due to the differences in the nature of implant-tissue attachment. Peri-implant probing may provoke a non-specific bleeding that is unrelated to the amount of inflammation in peri-implant tissues, moreover Ericsson and Lindhe (1993) found BOP in a large number of healthy peri-implant sites.

Microbiological investigation has also been used to identify peri-implant sites harboring potentially pathogenic microbial species (Rams et al. 1984, Mombelli et al. 1987). However, as in periodontitis, attempts to relate microbiological findings to the clinical course of peri-implantitis have not been successful in evaluating the presence of active disease or the risk of future disease progression (Fiorellini et al. 2000).

Overall, diagnosis of peri-implant disease is often based on patients' complaints and subjective clinical findings that may suggest peri-implant pathology (Mombelli & Lang 1994). Traditional measures have clear limitations as prognostic indicators for implant success. Moreover, in asymptomatic patients it is difficult to determine if the present clinical and radiographic findings represent active disease or evidence of past disease activity. This determination is critical for the proper management of the peri-implant tissue. Radiographic evidence of disease (present or past) requires two time-points to indicate changes. It is clear that establishing the diagnosis of peri-implant disease is lacking in objective parameters.

The analysis of peri-implant sulcus fluid

In the last decades, periodontal research has focused on the analysis of gingival crevicular fluid (GCF) with the aim of identifying potential host markers, which may permit more objective diagnosis of disease activity and prognosis of future disease. A number of markers, such as neutrophil elastase, prostaglandin E_2, β-glucuronidase, collagenase, alkaline phosphatase, aspartate aminotransferase and others, have been shown to be altered in the presense of periodontal disease.

Apse et al. (1989) observed that the volume of GCF in natural teeth and the volume of peri-implant sulcus fluid (PISF) in implant sites did not differ, and that the features of inflammation seem to be the same around teeth and implants.

Peri-implant sulcus fluid analysis may help in detecting early metabolic and biochemical changes not readily discernible; different biochemical markers in PISF have been widely studied in recent years. Last et al. (1995) tested for the glycosaminoglycan (GAG) content in PISF and detected two GAGs (hyaluronic acid and chondroitin 4-sulfate). They reported that increased GAG levels were assotiated with bone resorption. Panagakos et al. (1996) reported that elevated levels of interleukin (IL)-1α and IL-1β were associated with implants with peri-implantitis and this has been corroborated by Aboyoussef et al. (1998). Boutros et al. (1996) found that neutral protease levels were higher at the inflamed implant sites compared to the non-inflamed sites. Teronen et al. (1997), after analyzing PISF from healthy dental implants and implants with vertical bone loss, reported that matrix metalloproteinase-8 (MMP-8) activity was higher in PISF of implants with vertical bone loss compared to PISF of healthy implants, that is in agreement with Ma et al. (2000). Fiorellini et al (1997, 2000) investi-

gated aspartate aminotransferase (AST) activity in PISF and the authors found a statistically significant association of increased AST activity with positive BOP index, increased probing depth and increased GI. Boutros et al. (1996) reported that α-glucuronidase levels were significantly higher at failing implants when compared to healthy ones. In a recent study, Schubert et al. (2001) showed a clear correlation between the level of α-glucuronidase and bone loss. Boutros et al. (1996) demonstrated also significantly higher MPO levels (a marker of OxS) at failing implants compared with successful sites. The study of Adonogianaki et al. (1995) showed that absolute amounts of immunoglubulin G were higher in PISF from inflamed than healthy sites.

Although promising results have been achieved with these assays, none of these tests have been developed for routine use. Therefore more studies are needed to be performed in order to find reliable markers for peri-implant diagnosis and to determine the risk of developing peri-implant disease (Ozmeric 2003).

2.2. Inflammatory mediators in PISF and saliva

Cytokines, polypeptides with a wide spectrum of inflammatory, metabolic and immunomodulatory properties, are produced by a variety of cells, including the macrophage/monocyte system, dendritic cells, lymphocytes, neutrophils, endothelial cells and fibroblasts (Arai et al. 1990, Callard et al. 1999). As a consequence, cytokines, together with their receptors form a network of high complexity that is under tight biological control, including positive and negative feedback by the cytokines themselves. It is well known, that immunity depends on two major types of specific immune responses, termed cellular and humoral responses. The balance between them is regulated by factors associated with antigen-presenting cells (APC) and by cytokines produced by CD4+ T-helper (Th) cells, which can be divided into Th1 and Th2 subsets, with contrasting cytokine profiles (Mosmann et al. 1996). Several studies have demonstrated that some cytokines, such as IL-12, IL-1β, interferon (IFN)-γ, IL-6, and tumor necrosis factor (TNF)-α, are involved in Th-1 immune responses and induce mainly cell-mediated immunity. In contrast, IL-4, IL-5, IL-10 and IL-13 are involved in Th-2 immune responses and promote humoral immunity due to the production of B cell growth and differentiation factors (O'Garra 1998, Van der Broek et al. 2000, Belardelli & Ferrantini 2002). The major cytokine products of Th1 and Th2 cells are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype. Thus, INF-γ selectively inhibits the proliferation of Th2 cells, while IL-4 and IL-10 inhibit cytokine synthesis by Th1 cells (Mosmann & Sad 1996, Essner et al. 1998). Moreover, IL-4 suppresses the synthesis of proinflammatory cytokines, including IL-1 and TNF-α, which induce several events associated with inflammation, tissue

destruction, bone resorption and the production of matrix metalloproteinases and prostaglandin E_2 (Yucel-Lindberg et al. 1999). One of the major sources of IL-1β and TNF-α in inflamed tissue are monocytes/macrophages. INF-γ and IL-2 are produced mainly by Th1 cells, while IL-4 and IL-10 by the Th2 subset. All these cytokines play a crucial role in immune and inflammatory responses, and the outcome of infection may be attributable to the relative balance between them.

In contrast with the specifity of cellular and humoral immunity, the acute-phase changes are nonspecific and occur in response to many conditions (Jaje et al. 1997). The acute-phase reaction represents an early and highly complex reaction of the organism to a variety of injuries such as bacterial, viral or parasitic infection, mechanical or thermal trauma, ischaemic necrosis, or malignant growth (Koi 1996). The purpose of these non-specific responses is to restore homeostasis and to remove the cause of disturbance. Cytokines, related to the acute phase response can be divided into two main groups: 1) pro-inflammatory cytokines initiating or enhancing the cascade of events (interleukin-6 type cytokines such as IL-6, leukemia inhibitory factor, IL-11, oncostatin M, ciliary neurotrophic factor and cardiotrophin-1; and other like TNF-α, IL-1, IFN-γ and IL-8), which are held responsible for the main systemic features of acute–phase response in a variety of tissues; and 2) anti-inflammatory cytokines downregulating the acute-phase response (IL-10, IL-4, IL-13 and transforming growth factor β) (Koi 1996).

Recent evidence indicates that inflammatory cytokines, released by the monocytes and macrophages in response to bacterial products such as lipopolycaccharide and endotoxin, are responsible for the breakdown of the periodontium in periodontitis (Jandinski et al. 1991, Stashenko et al. 1991a). Many studies (Stashenko et al. 1991b, Gore et al. 1998, Figueredo et al. 1999) have shown that IL-1β is present at elevated levels in the GCF and in tissue from periodontal pockets. TNF-α, a cytokine with functions similar to those of IL-1β, has been detected in a very low levels from sites with periodontitis. Baqui et al. (2000) showed that levels of IL-6 are also increased in GCF from periodontitis sites. These cytokines stimulate bone resorption, prostaglandin synthesis and protease production by many cell types including fibroblasts and osteoblasts (Dewhirst et al. 1985, Beutler & Cerami 1986, Billingham 1987, Lorenzo et al. 1987, Tatakis et al. 1988). IL-10 suppresses inflammatory responses, including the expression of IL-1, and has been shown to inhibit bone resorption *in vitro* (Fiorentino et al. 1991, Wang et al 1995, Kucharzik et al 1996, Sagawa et al. 1996, Hart et al 1996) and *in vivo* (Sasaki et al. 2000).

Although extensive research has been done in the area of periodontal inflammatory mediators, few have studied the role of the host immune response in peri-implantitis and peri-implant mucositis. Significantly elevated PISF levels of IL-1β have been determined in mouths with failing implant sites compared to mouths with healthy control implants (Panagakos et al. 1996, Salcetti et al. 1997, Aboyoussef et al. 1998).

2.3. Oxidative stress

As any inflammation has an OxS-expressed character, a short overview about oxidative stress will be presented.

Concept of oxidative stress

In healthy state there is a dynamic balance between pro-oxidants and antioxidant defense system (Kehrer 1993, Zilmer et al. 1994, Halliwell 1996). In certain situations, such a balance becomes disturbed in favor of the pro-oxidants. Thus, oxidative stress (OxS) could be defined as a situation of profound disturbance in the pro-oxidant and anti-oxidant balance in favour of the former (Kehrer 1993, Pincemail et al. 1996). Prolonged high-grade OxS leads to tissue damage (Kehrer 1993, Zilmer et al. 1994, Halliwell 1996, Sies 1997).

Formation of reactive oxygen species and consequences of their production

Under normal conditions about 95% of molecular oxygen in biosystems undergoes tetravalent reduction by efficient intracellular systems, such as the respiratory chain to produce ATP. However, 3–5% escapes this pathway through other routes, and this results in formation of reactive oxygen species (ROS) (Gutteridge & Halliwell 1994). Reactive oxygen species are atoms, ions or molecules with one or more electrons in unpaired spin, which increases their reactivity. They may have either free radical nature (superoxide anion, hydroxyl radical, peroxyl and alkoxyl radicals) or not (hydrogen peroxide, singlet oxygen, hypochlorous acid) (Kehrer 1993). All aerobic cells, and especially polymorphonuclear leucocytes (PMN), are capable of producing ROS, that is the PMN's main defence mechanism (Halliwell & Gutterlidge 1999). Numerous intracellular sources of ROS have been identified, which include the mitochondrial electron transport chain, the action of various soluble or membrane-bound enzymes (especially xanthine oxidase, but also lipoxygenase, prostaglandin synthetase, NADPH oxidase), as well as reactions involving transition metals, particularly iron and copper (Kehrer 1993, Curello et al. 1995).

In vitro studies demonstrated that elevated ROS are highly cytotoxic agents (Romaschin et al. 1990**,** Ytrehus & Hegstad 1991**,** Kuzuya et al. 1993). Reactive oxygen species are able to initiate a lipid peroxidation, to inactivate proteins, to promote DNA injury, and to degrade carbohydrates (Ytrehus & Hegstad 1991**,** Coetzee et al. 1994). In the oral cavity, increased ROS production may lead to the destruction of connective tissue via the inhibition of antiproteases (Yamalik

et al. 2000). Overall, under conditions of high-grade OxS all types of biomolecules may be targeted by ROS.

Protection against ROS

Protection against ROS is offered by the complex and integrated action of antioxidants. The scavenger-like substances can be divided into enzymatic and non-enzymatic groups. The primary scavenging enzyme is cellular superoxide dismutase (SOD), which catalyzes the dismutation of superoxide to hydrogen peroxide (H_2O_2) (Kehrer 1993, Halliwell 1996). Hydrogen peroxide is further scavenged by other antioxidant enzymes, catalase and glutathione peroxidase. The latter enzyme's activity is closely linked to glutathione (GSH), which is a central non-enzymatic intracellular antioxidant (Zilmer & Zilmer 1994). In the extracellular space, the protection of biomolecules against ROS is provided by simultaneous action of non-enzymatic antioxidants like ascorbate, α-tocopherol, carotenoids, metal binding proteins transferrin and ceruloplasmin, and also by free sulphydryl groups in proteins (Halliwell & Gutteridge 1990, Kehrer 1993). Independent of their intra- or extracellular presence, the antioxidant system is best characterized by its overwhelming complexity (Zilmer et al. 1994, Pincemail et al. 1996) and consequently relative and short-term lack of one component of this system does not always result in changes in total antioxidant capacity of an organism, and has only minor (patho)physiological consequences. However, a decrease in total antioxidant capacity (potency) may result in a shift of the balance between pro- and antioxidants towards the former that consequently leads to OxS and possible tissue damage.

Oxidative stress and pathophysiological conditions

An increased level of ROS has been suggested as a participant in more than 100 pathophysiological conditions that range from general inflammatory responses to retinopathy, cardiovascular diseases and cancer (Lunec 1990, Kehrer 1993, Zilmer et al. 1994, Droge 2003, Byrne et al. 2003, Casetta et al. 2005, Dryden et al. 2005, Galli et al. 2005, Okayama 2005). With respect to oral disorders it is believed that they contribute to the pathogenesis of periodontitis (Battino et al. 1999). Such a wide list of diseases raises the question of a common pathophysiology, but it is important to notice that although ROS may initiate a series of damaging biochemical events, they are not necessarily directly responsible for the final tissue dysfunction.

Polymorphonuclear neutrophils: a key role in periodontal diseases

The periodontal diseases are comprised of a group of inflammatory conditions that result in the destruction of the supporting structures of the dentition. Periodontal diseases are infectious diseases. The etiology is specific gram-negative micro-organisms, such as *Porphyromonas gingivalis* and *Bacteroides forsythus* in the case of chronic periodontitis, and *Actinobacillus actimnomycetemcomitans* in the case of Localized Aggressive Periodontitis. While the etiology of periodontitis is bacterial, it is becoming clear that the pathogenesis of disease is mediated by the host response (Van Dyke & Serhan 2002).

Neutrophils (PMN) are the first line of host defence by their ability to phagocytize microbes. They can also give rise to PMN-dependent vascular injury and contribute to increased vascular permeability, edema, and further release of chemoattractants.

However, in addition to the direct involvement of neutrophils in the defense against pathogens, the neutrophils' role in mediating tissue destruction in inflammatory diseases is also a significant parameter in pathogenesis (Weiss 1989, Baumann & Gauldie 1994). Neutrophil-mediated tissue injury plays an important role in the pathogenesis of several significant diseases (Van Dyke & Serhan 2002).

Until recently, defects associated with neutrophil functions were believed to predispose individual to infection. However, there is a growing body of evidence suggesting that the neutrophil abnormalities in periodontal disease may be the result of a chronic hyperactivated or "primed" state of the periodontal neutrophil. Under this paradigm shift, the neutrophil is considered not hypofunctional or deficient, but hyperfunctional, and it is an excess activity (accompanied by potent production of reactive species) and the release of toxic products from the cell that are responsible, in part, for the tissue destruction in chronic periodontal inflammation (Van Dyke & Serhan 2002).

Reactive oxygen species produced by PMNs can disrupt biomolecules, such as proteins, lipids, carbohydrates and nucleic acids (Badwey & Karnovsky 1980). These ROS include superoxide and hydroxyl radicals and hydrogen peroxide. Superoxide anion (O_2^-) is the initial product generated by the reduction of molecular oxygen (O_2) by NADPH oxidase in PMN. This process is summarized as a respiratory burst characterized by a potent increase in cellular oxygen consumption, activation of several G proteins (proteins involved in second messenger cascades and located on the cell surface), which begins a cascade of events that leads to the cell response (Badwey & Karnovsky 1980). Superoxide anions are converted into hydrogen peroxide by SOD. Neutrophils also contain myeloperoxidase, which catalyzes the conversion of hydrogen peroxide into hypochlorous acid (HOCl). Hypochlorous acid is evidently the principle product responsible for the cytotoxicity of neutrophils (Badwey &

Karnovsky 1980, Weiss 1989, Baumann & Gauldie 1994). This cytotoxicity derives from the resultant production of powerfully oxidizing chloramines (Entman & Smith 1994). In addition to their direct oxidative attack, ROS can also modulate various cellular activities that are important mediators in the sequence of events leading to tissue injury (Gasic et al. 1991).

Myeloperoxidase is also suggested to have a significant contribution to protease activity. The interaction between proteases and MPO is explained by the activation of proteases via inhibition of antiproteases by the MPO system. Hypochlorous acid, as the product of an MPO-catalyzed reaction, inactivates α_1-protease inhibitor by oxidizing a methionine residue essential for the action of α_1-protease inhibitor. Due to the inhibition of antiprotease shield by hypochlorous acid, MPO can provide appropriate conditions for most proteases, particularly when the concentration of ascorbate (a scavenger of hypochlorous acid) has been depleted. The activation of latent proteases and inhibition of antiproteases change the protease/antiprotease balance and the impairment of this balance is considered as an important step in connective tissue breakdown, therefore these two enzymes seem to be functionally related**.** In summary, elevated MPO level can be considered as an adequate marker of inflammation (Blake & Ridker 2002; Brennan & Hazen 2003; Himmelfarb 2004; Shishehbor & Hazen 2004; Wu 2005).

Neutrophil-mediated tissue damage in the periodontium was first demonstrated by Deguchi et al. (1990). Similar findings were reported for neutrophil and gingival epithelial cell interactions (Altman et al. 1992), where it was demonstrated that human neutrophils could lyse epithelial cells in an *in vitro* model mediated by MPO-hydrogen peroxide interactions. In addition to the direct deleterious activities of neutrophils on host periodontal tissues, superoxide generation was shown to be elevated in both resting and stimulated aggressive periodontitis neutrophils (Van Dyke et al. 1986).

In summary, hyperfunctional neutrofils produce excessive amounts of ROS, thus inducing a high-grade OxS, which in turn is associated with any inflammatory state (Stocker & Keaney 2004).

It may therefore be hypothesized that high-grade OxS has implications on the pathogenesis of peri-implant disease, similar to periodontal disease.

2.4. Oxidative stress-targeted antioxidant management

The aim of antioxidant adjuvant therapy is to diminish/suppress/avoid/high-grade OxS. Antioxidants are substances that delay or inhibit oxidation of substrate. The principal non-enzymatic antioxidants are vitamins E, Q and C.

Vitamins essential for antioxidant management

Ubiquinone (Coenzyme Q10 or vitamin Q)

Ubiquinones, substances with vitamin-like properties, are found in all living cells in animals and plants, where they have important biofunctions (Brunmark & Cadenas 1989). The human organism synthesizes a limited quantity of the specifically human ubiquinone, coenzyme Q10, itself; additional coenzyme Q10 is consumed as a part of the diet. Coenzyme Q10 plays a central role in the cells during the production of metabolic energy (ATP). Furthermore, it functions as an antioxidant in the body's lipid phases in much the same manner as vitamin E and various carotenoids. Finally, coenzyme Q10 posesses membrane-stabilizing properties.

Various studies have shown that individuals with specific disease conditions have levels of coenzyme Q10 that are significantly lower level than in healthy individuals (Folkers 1992). For this reason, it has been hypothesized that coenzyme Q10 can be employed both as one of the therapeutic substances and a preventative agents in relation to a number of diseases.

In case of periodontal disease, research has shown a positive clinical effect from both oral administration of coenzyme Q10 (Nylander et al. 1996a) and topical application directly onto the gingiva (Nylander et al. 1996b). Q10 has been shown to reduce bleeding from infected gingiva (Nylander et al. 1996b). Moreover, it has been reported that the Q10 concentration decreased from normal values in sites with periodontitis (Lunn et al. 1997).

Vitamin E (α-tocopherol) and vitamin C

Vitamin E is one of the most important lipid-soluble antioxidant vitamins. It has a phenolic -OH group, which is responsible for its anti-oxidant activity and a phytyl side-chain that favors its insertion into the cell membrane lipid bilayer. According to the anti-oxidant theory, vitamin E inhibits lipid peroxidation, scavenging peroxyl radicals much faster than the radicals can react with adjacent fatty acid side-chains or with membrane proteins (Gutteridge and Halliwell 1994). The peroxyl radical is converted to a lipid peroxide and α-tocopherol to an α-tocopheroxyl radical which, in turn, is alternatively regenerated to α-tocopherol by ubiquinol ($CoQH_2$: the reduced form of coenzyme Q), GSH, or, mainly, by ascorbic acid. The latter interaction generates an ascorbic acid radical (a fairly unreactive species), which, reacting with another ascorbic acid molecule, yields ascorbate and dehydro-ascorbate, or it can be reduced by a CoQ-dependent dehydrogenase (Navarro et al. 1995, Villalba et al. 1995). The antioxidative protection afforded by vitamin E occurs in the body's lipid phase in various structures in the cells, e.g. in fatty acids in the cell membranes and in LDL-cholesterol molecules (Esterbauer et al. 1991).

Gingival vitamin E concentrations decrease in proportion to the severity of pocket depth and gingival and bleeding indices (Fujikawa 1983).

Ascorbic acid is the only endogenous antioxidant in plasma that can completely protect against peroxidative damage induced by aqueous peroxyl radicals and the oxidants released from activated neutophils (Brown & Jones 1996). Ascorbic acid seems to protect against peroxidation indirectly, by interacting with α-tocopherol (Buettner & Jurkiewicz 1996). There would appear to exist a concerted action of vitamins E and C, in which ascorbic acid regenerates vitamin E, thus maintaining its serum value at constant level. When ascorbic acid is depleted, no regeneration of vitamin E is possible, and a decrease in its concentration is observed (Gutteridge & Halliwell 1994, Roberfroid & Buc Calderon 1995). Chemically, ascorbic acid is an excellent reducing agent (Buettner & Jurkiewicz 1996), and most of its antioxidant properties are ascribed to this feature.

α-Lipoic acid

α-lipoic acid (ALA) is a natural dithiol compound derived from octanoic acid. In humans, ALA is synthetized by the liver and other tissues, and functions as a co-factor within pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Schmidt et al. 1994). ALA is both water and lipid soluble, and therefore is widely distributed in plants and animals. In addition, ALA and its reduced dithiol form, dihydrolipoic acid (DHLA), are powerful antioxidants, their functions described by Biewenga et al. (1997) include: 1) quenching of reactive oxygen species, 2) regeneration of exogenous and endogenous antioxidants such as vitamins C and E, and glutathione, 3) chelation of metal ions, and 4) reparation of oxidized proteins. In most cells containing mitochondria, ALA is reduced by an NADH-dependent reaction with lipoamide dehydrogenase to form DHLA. In cells that lack mitochondria ALA can be reduced to DHLA via NADPH with glutathione and thioredoxin reductases (Jones et al. 2002). ALA is used as a potent antioxidant, as a detoxication agent for heavy metal poisoning, and has been implicated as a means to reduce age-associated cognitive decline (Rahimi et al. 2005). Recent evidence shows that ALA has blood lipid modulating characteristics, thus protecting against LDL oxidation and modulating hypertension. Therefore, ALA is believed to be a protective agent against risk factors associated with cardiovascular disease (Wollin & Jones 2003).

Microminerals (selenium, zinc, manganese) related to antioxidativity

Selenium

Selenium is an essential co-factor in cellular antioxidants such as glutathione peroxidase that help to protect tissues against oxidative damage. It is hypothesized that selenium levels drop in response to inflammation and that supplementation may have anti-inflammatory effects (Rennie et al. 2003). In immune cells, the major function of selenium appears to control excessive production of peroxidative substrates and it may also down-regulate cytokine signaling (Rennie et al. 2003).

Zinc

Together with copper, zinc acts an essential cofactor of the antioxidant enzyme superoxide dismutase (Cu, Zn-SOD). Zinc supplements seem to play an indirect antioxidative roll by taking part in the activity of the SOD-enzyme. There have also been reports of an antioxidative roll for zinc independent of the SOD-enzyme (DiSilvestro 2000). The other possible indirect antioxidant roles of zinc are as follows: 1) protection against vitamin E depletion; 2) stabilization of membrane structure; 3) restriction of endogenous free radical production; 4) maintenance of tissue concentrations of metallothionein, a possible scavenger of free radicals. Recent evidence shows that zinc deficiency influences OxS status in patients with diabetes (DiSilvestro 2000) and rheumatoid arthritis (Rennie et al. 2003).

Manganese

Manganese (Mn) is the cofactor for catalases, mitochnondrial superoxide dismutase and peroxidases that defend against ROS (Yocum & Pecoraro 1999). Although there are many examples of Mn participating in enzyme functions, there are also descriptions of this metal acting catalytically as an antioxidant by association with anions including phosphate, and metabolic intermediates such as lactate or malate (Archibald & Fridovich 1981, Archibald & Fridovich 1982a, Archibald & Fridovich 1982b, Archibald & Duong 1984).

Manganese fulfils several different roles in biological systems, acting as a simple Lewis acid catalyst or switching between different oxidation states (Yocum & Pecoraro 1999). Its role as an antioxidant in low molecular weight complexes is likely to involve redox reactions between the Mn(II) and Mn(III) states, although much of the *in vivo* chemistry of Mn and its role as an anti-oxidant remains to be resolved (Horsburgh et al. 2002).

Experiments *in vitro* have shown that Mn(II) can act catalytically as a scavenger of either superoxide (O_2^-) or hydrogen peroxide (H_2O_2) as shown

below. These reactions are not balanced in charge, as the precise reactions and participating ligands are not yet clear.

Mn(II) scavenging of superoxide (Yocum & Pecoraro 1999)

$$Mn(II) + O_2^- + 2H^+ \rightarrow Mn(III) + H_2O_2 \rightarrow Mn(II) + O_2 + 2H^+$$

As an alternative to the above mechanism, the H_2O_2 formed during O_2^- scavenging by Mn(II) can reduce the Mn(III) formed as shown or it can be dismutated by catalase with subsequent reduction of Mn(III) by a cellular reductant such as cysteine or glutathione.

Mn(II) scavenging of hydrogen peroxide

$$Mn(II) + H_2O_2 \rightarrow\rightarrow Mn(II) + O_2 + 2H^+$$

The exact mechanism for catalytic scavenging of H_2O_2 is less clear and is thought to involve intermediate steps as indicated by the arrows; the reaction intermediates O_2^- and $OH^\bullet$ have been observed *in vitro* (Stadtman 1990).

Thus, one part of this study is carried out to test the hypothesis that multicomponental antioxidant complex (coenzyme Q10, vitamin E natural isomer, vitamin C, lipoic acid, selenium, zinc and manganese) could have an impact on treatment and prevention of inflammatory diseases of tooth or implant supporting structures in the mouth.

3. AIMS OF THE STUDY

The overall aim of this study was to investigate concurrently inflammation- and OxS-related indices in the patients with peri-implant disease to determine their role in the etiology and pathogenesis of peri-implant disease.

At first, we hypothesized that high-grade OxS could be associated with inflammatory diseases of tooth and/or implant supporting structures. To test this hypothesis, the first pilot study was designed to evaluate the effect of antioxidant management in suppression of inflammatory diseases of tooth supporting structures in the mouth. If this hypothesis could be proven, we could go further to study specific aspects related to high-grade OxS in association with inflammatory diseases of tooth and/or implant supporting structures.

The specific objectives were:

1. To evaluate the effect of dietary antioxidant management in supression of inflammatory diseases of tooth supporting structures in the mouth.
2. To estimate the possible role of high-grade oxidative stress in the pathogenesis of peri-implant disease.
3. To test the correlation of the levels of biochemical markers of inflammation in PISF with traditional clinical parameters in partially edentulous patients with peri-implant disease.
4. To test the correlation of the levels of biochemical inflammatory markers in saliva with traditional clinical parameters in totally edentulous patients with peri-implant disease.
5. To estimate possibility to use biochemical markers of inflammation in early-stage diagnostics of peri-implant disease.

4. PART I: THE EFFECT OF MULTICOMPONENTAL ANTIOXIDANT SUPPLEMENTATION IN PATIENTS WITH PERIODONTITIS

Increased free-radical activity may be implicated in the pathogenesis of periodontal disease and knowledge of the response to micronutrient supplementation of oral/blood antioxidant systems is limited in literature. Accordingly, this study was initiated to assess glutathione and total antioxidant status, lipid peroxidation markers in patients with advanced periodontitis before and after antioxidant therapy for a period of 5 weeks. Saliva was used to measure oxidative stress markers.

4.1. Patients and methods

4.1.1. Patient population

Nine patients (5 males, 4 females) with advanced periodontitis (test group) and a control group of nine dentists (2 males, 7 females) were randomly selected and included in the study. The age of the subjects in the test group varied between 47 and 69 years and that of the controls between 25 to 51 years. The diagnosis of advanced periodontitis was based on the clinical criteria of Carranza & Newman (1996). All the patients were non-smokers. The patients were examined twice, before and after supplementation with antioxidant cocktail that included 60mg of coenzyme Q_{10} and ascorbic acid, 75mg of vitamin E (D-alpha-tocopherol), 0.050mg of selenium, 2.5 mg of manganese and 10mg of zinc per day and 100mg of alpha-lipoic acid every second day.

4.1.2. Gingival condition

The gingival condition of all subjects was assessed by detecting bleeding on probing (BOP) index, registering the PPDs at four sites (mesial, distal, buccal, lingual) of all teeth, evaluating the mobility of teeth and evaluating the furcation invasions.

All clinical parameters were recorded by a single examiner. Calibrated perio-probe with constant probing pressure of 20…25 g (Hawe Click-Probe) was used (Karayannis et al. 1992, Lang et al. 1994).

4.1.3. Saliva sampling

Resting saliva was collected at 5...6 p.m. (3…4 hours after eating, the subjects were requested to refrain from all oral hygiene procedures in the morning and during the day) into plain glass vials and stored frozen at –20°C prior to use (Thylstrup & Fejerskov 1994). Before the measurement the samples were unfrozen and centrifuged for 15 minutes at 3000 rpm and the supernatant removed for immediate analysis.

4.1.4. Chemical assays

The assessment of OxS in saliva was performed via assay of reduced and oxidized glutathione (GSH and GSSG, respectively), markers of lipid peroxidation as thiobarbituric acid reactive substances and lipid hydroperoxides (TBARS and LOOH, respectively) and total antioxidative status and total antioxidative protection (TAS and TAP, respectively).

Glutathione was measured by using an enzymatic method modified by Griffith (1980) and described by Bhat et al. (1992). The glutathione redox ratio was expressed as GSSG/GSH.

TBARS level of saliva was measured as described earlier (Ohkawa et al. 1979, Ristimäe et al. 1999). Briefly, samples were incubated with 0.475 M Fe^{2+} at 37°C for 30 min. After incubation, butylated hydroxytoluene (0.25%) was added to the samples, treated with acetate buffer (pH 3.5) and heated with TBA solution (1%, 80°C, 70 min). Then the samples were cooled and acidified (5 M HCl). After extraction with cold butanol, samples were centrifuged and absorbance of butanol was measured at 534 nm. LOOH level was measured by using commercial available kits (K-assay LPO-CC: Kamiya Biomedical Company, Seattle, WA, USA).

Salivary TAS was estimated by the ABTS assay (Rice-Evans & Miller 1994), which involves the interaction of the ferrylmyoglobin radical, produced from activation of metmyoglobin with the phenothiazine compound ABTS. In the presence of antioxidants, the absorbance of the ABTS radical cation is inhibited to an extent and on a timescale dependent on the total antioxidant status of sample.

Saliva TAP was measured as described earlier (Ristimäe et al. 1999) assessing the ability of the sample to inhibit linolenic acid (LA) peroxidation. Briefly, standard of LA in isotonic saline (0.4 ml), sodium dodecyl sulphate (0.015 ml), and sample were incubated in the presence of 0.2 mM iron at 37°C for 60 min. Butylated hydroxytoluene (0.25%) was added, then the samples were treated with acetate buffer (pH 3.5), heated with thiobarbituric acid solution (1%, 80°C, 35 min) and assessed for TBARS. The results were

expressed as percentage of inhibition of LA peroxidation induced by serum samples.

All determinations of OxS stress markers were performed in triplicate.

4.1.5. Statistical analysis

Numerical data are presented as mean values ±SEM. Differences were calculated by ANOVA. Interactions between several independent variables were then examined with multiple regression analysis. $P< 0.05$ was regarded significant.

4.2. Results

The gingival condition of the patients with advanced periodontitis notably improved from initial examination to 5 weeks after supplementation with antioxidant cocktail. There was a statistically significant decline in BOP index in the patients group after supplementation with antioxidant cocktail (52.7 ± 24.0% before *vs* 30.4 ± 11.9% after, $p<0.05$). However, the BOP index values were still higher than those in the healthy control group (3.1 ± 6.3%, $p<0.05$).

The most significant differences were found concerning glutathione status (Table 1). Glutathione redox ratio (GSSG/GSH), known as a strong marker of intracellular OxS (Anderson & Luo 1998, Halliwell & Gutteridge 1999), was 2.30 and 0.46 before treatment and after treatment, respectively (Fig. 1).

Table 1. The oxidative stress markers of saliva samples in healthy subjects (control group) and in patients with advanced periodontitis before and after antioxidative therapy (n=9)

	TAS mmol/l	TAP %	GSSG µg/ml	GSH µg/ml	TBARS nmol/l	LOOH nmol/ml
Healthy	0.38 ± 0.05	36.44 ± 1.39	0.07 ± 0.01	1.67 ± 0.18	0.50 ± 0.23	1.01 ± 0.35
Patients before treatment	0.39 ± 0.10	37.38 ± 1.97	2.92 ± 0.76 *	1.50 ± 0.33	0.43 ± 0.10	2.27 ± 0.30
Patients after treatment	0.35 ± 0.11	32.71 ± 1.30	1.59 ± 0.38	4.05 ±1.10 **	0.50 ± 0.12	0.70 ±0.25 ***

*: $P < 0.001$ (healthy vs. before treatment); **: $P < 0.05$ (before treatment vs. after treatment); ***: $P< 0.001$ (before treatment vs. after treatment)

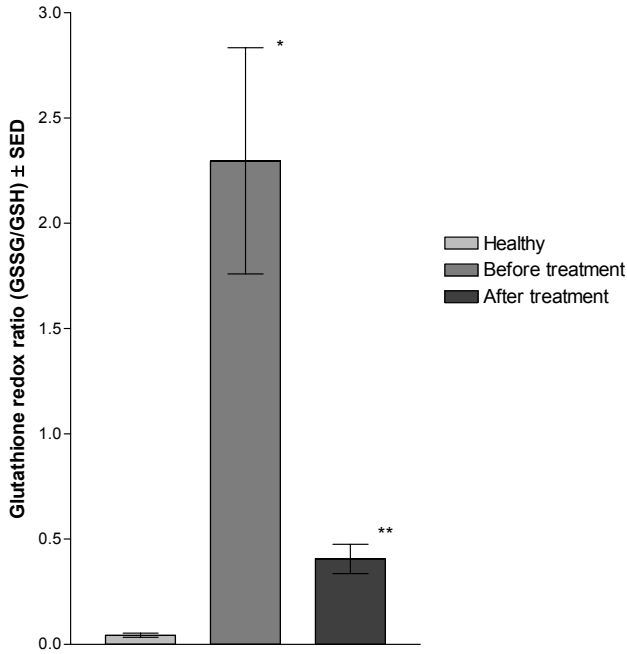

Figure 1. The glutathione redox ratio (GSSG/GSH) in saliva from healthy subjects and in saliva of patients with advanced periodontitis before and after antioxidant treatment.

* $p < 0.001$ (healthy vs before treatment); ** $p < 0.001$ (before treatment vs after treatment)

LOOH was significantly increased in patients with advanced periodontitis and its level returned to normal after antioxidant therapy (Table 1).

The level of TBARS (it is less specific marker of LP) was to a certain extent increased in patients group and its level remained unchangeable after the treatment (Table 1).

The findings (Table 1) indicated that both saliva TAS and TAP were practically similar in healthy person and in patients with advanced periodontitis.

4.3. Discussion

In this study glutathione, total antioxidant status and lipid peroxidation markers were measured in patients with advanced periodontitis before and after antioxidant therapy and in non-diseased control group. Saliva was used to measure oxidative stress markers.

The most significant differences were found in glutathione status (Table 1). Glutathione redox ratio (GSSG/GSH) is known as an adequate marker of intra-

cellular OxS (Anderson & Luo 1998, Halliwell & Gutteridge 1999). Glutathione redox ratio in diseased group was 2.30 before treatment and 0.46 after treatment, while in healthy control group it was 0.04 (Fig. 1). This means, that patients with advanced periodontitis have high-grade cellular OxS as compared to the healthy control group, and systemic antioxidant manipulation may have contributed to the decline of elevated OxS. It is noteworthy that the reduction of the glutathione redox ratio is due to the increase of the amount of the reduced glutathione and decrease of the GSSG level (Table 1), the latter have toxic influence at high levels.

Therefore, the change in the glutathione status may reflect the level of cellular OxS that may be correlated with the periodontal disease state. Glutathione has been shown to provide protection against oxidative stress in the tissues and biological fluids (Anderson & Luo 1998). Previous experimental study (Khmelevski et al. 1985) has demonstrated that during severe inflammation there is a considerable increase of the glutathione sulfhydryl groups in the gingival tissue in patients with periodontitis. After vitamin therapy (vitamin A, E and K were used) this parameter was normalised.

It is known that systemic high-grade OxS is characterized by elevated lipid peroxidation (LP) and total antioxidative status. To evaluate LP level it is necessary to assay at least two different indices. The study showed that lipid hydroperoxides (LOOH) was significantly increased in patients with advanced periodontitis and their level returned to normal after antioxidant supplementation (Table 1). The level of tiobarbituric acid reactive substances (TBARS), a less specific marker of LP, was to a certain extent increased in patients group and its level remained unchangeable after the treatment (Table 1). The findings (Table 1) indicate that both saliva TAS and TAP were practically similar in healthy persons and in the patients with advanced periodontitis.

The findings indicate the existence of high-grade cellular oxidative stress in patients with advanced periodontitis. It seems that imbalance in the oxidant-antioxidant status may be one of the pathogenic factors in the development of periodontal disease. Supplementation with antioxidative cocktail, lasting for 5 weeks, lowered oxidative stress in the oral cavity, reduced inflammation and improved bleeding index.

5. PART II: CORRELATION OF PERI-IMPLANT HEALTH AND MYELOPEROXIDASE LEVELS

The purpose of the study was to evaluate the correlation of MPO levels with traditional periodontal clinical parameters around dental implants including peri-implant PPD, GI and BOP, since MPO has been associated with destruction of periodontal tissues.

5.1. Patients and methods

5.1.1. Patients

The population for this cross-sectional study consisted of 24 subjects (41–63 years of age; 15 male and 9 female), randomly selected from a group of patients treated with implants (Ankylos® dental implants, DentsplyFriadent, Mannheim, Germany) and receiveing maintenance care at the Tallinn Dental Clinic. All the patients were non-smokers. The mean time in function was 69.2 ± 21.6 months (ranged between 24 and 94 months).

Prior to the start of the study the subjects gave their informed consent. Inclusion criteria were: (1) presence of at least 1 endosseous dental implant restored with appropriate prosthesis, (2) no history of antibiotic treatment for 3 months prior to the study, (3) no history of medical conditions that required antibiotic prophylaxis and (4) no history of chronic corticosteroid use.

A complete oral examination was given prior to performance of the MPO assay and measurement of clinical parameters.

5.1.2. Criteria for healthy and diseased peri-implant tissues

Studied implant sites were categorized on the basis of PPD, GI and BOP into two different groups as follows: implants with inflammatory lesions (peri-implantitis and peri-implant mucositis) and healthy implants. The criteria have been referred to in previous reports (Pontoriero et al. 1994, Mombelli & Lang 1998). Briefly, peri-implantitis is defined as an inflammation process around implants with loss of supporting bone and peri-implant mucositis is defined as reversible inflammation of the soft tissues with no loss of supporting bone. According to these definitions we applied the clinical parameters as shown in Table 1.

Table 1. Clinical assesment and diagnosis of implants

Diagnosis	PPD	GI	BOP
Implants with inflammatory lesions	≥ 4 mm	≥ 1	1
Healthy implants	≤ 3 mm	0	0

PPD – probing pocket depth, GI – Gingival Index, BOP – bleeding on probing

5.1.3. Clinical examination

The clinical evaluations were performed by single examiner (S.L) and included assessment of peri-implant PPD (mm), GI (0,1,2 or 3) (Löe et al. 1963) and BOP (0 or 1). The PPD was measured to the nearest mm with a pressure-calibrated periodontal probe with a tip diameter of 0.5 mm and a probing force of 0.25 N (ClickProbe®, Hawe-Neos Dental, Bioggio, CH) (Karayannis et al. 1992, Lang et al. 1994).

5.1.4. Peri-implant sulcus fluid sampling

Since no definite data concerning the normal volume of PISF has been reported, the amount of PISF was represented as weight, not as the volume. For this purpose plastic vials for PISF sampling were weighed prior to assessment and placed into the transport vials (plastic vials of larger size) to avoid contamination and possible weight changes.

Sites selected for PISF sampling were isolated with cotton rolls. A gentle stream of air was directed parallel to the implant surface for 5 to 10 seconds to dry the area. Thereafter PISF was collected using micropipette from each implant site (from buccal crevice and from lingual/palatal crevice). The tip of micropipette was washed with sterile saline and dried before insertion into the crevice. Plastic vials for PISF collection were removed from transport vials prior to PISF collection. Collected PISF was transferred into plastic vials, which were placed to transport vials again and frozen at –70º C for subsequent laboratory analysis.

5.1.5. Biochemical analysis of peri-implant sulcus fluid

Transport vials containing PISF vials were unfrozen prior to measurement of MPO levels. Plastic vials containing peri-implant sulcus fluid were weighed again to obtain the weight of PISF. MPO levels were measured with Bioxytech® MPO Enzyme immonoassay (Oxis International, Portland, USA). The MPO-EIA method is an enzyme-linked immunoassay of the ELISA type. Samples were diluted in 150 µl of Sample Diluting Buffer. Thereafter samples were

incubated in the wells of a sectionable microplate, which have been coated with the monoclonal antibody to MPO. The MPO-Mab complex was labeled with a biotin-linked polyclonal antibody prepared from goat MPO-antisera. The final step of the assay was based on a biotin-avidin coupling in which avidin has been covalently linked to alkaline phosphatase. The amount of MPO in each sample was enzymatically measured upon addition of 4-nitrophenyl-phosphate (pNPP), by reading the microplate at 405 nm.

5.1.6. Statistical analysis

Student's *t*-test was used to compare mean PPD levels and mean MPO levels between healthy and diseased implants. Since MPO levels did not follow a normal distribution, a log-transformation was used. Geometric mean of the MPO levels together with 95% Confidence Interval (CI) were calculated separately for healthy and diseased implants, implants with different GI and BOP levels and for implants with different PPD.

For comparison of percentage of sites with GI > 1 and BOP=1 between healthy and diseased implants, Pearson $\chi 2$ test was used.

For visual comparison of the distribution of MPO values in different levels of clinical parameters, boxplots (on logarithmic scale) were used.

5.2. Results

5.2.1. Patient data and clinical results

Thirty-four implants in 19 patients (12 men and 7 women) showed signs of inflammation. A second group of 31 implants in 21 patients (13 men and 8 women) with healthy peri-implant tissues were used as controls.

Table 2 shows the clinical status of each group including PPD, GI and BOP. Statistically significant differences between the two groups were observed for all clinical parameters (P<0.0001).

Table 2. Clinical data from healthy and diseased implants.

	Healthy	Diseased	P
PPD (mean ±SD)	3.1 (0.4)	5.0 (1.6)	<0.0001
% sites with			
gingival inflammation (GI>1)	9.4	87.5	<0.0001
bleeding on probing (BOP =1)	9.4	90.6	<0.0001

PPD – probing pocket depth, GI – Gingival Index, BOP – bleeding on probing

5.2.2. Biochemical analysis of peri-implant sulcus fluid

Table 3 shows the total amounts (geometric mean with 95% CI) of MPO in peri-implant sulcus fluid of healthy and diseased groups. Significant differences at the level of P<0.0001 were found between the two groups.

Table 3. Total amounts (geometric mean with 95% CI) of MPO in healthy and diseased implants.

Status	MPO levels (geometric mean with 95% CI), ng/mg	% above 25 ng/mg
Healthy	8.5 (7.9, 14.3)	9.4
Diseased	122.8 (118.4, 370.3)	96.9

Figure 1 shows relationship between PPD and MPO activity. MPO activity is increasing with the increase of PPD. Figures 2 and 3 show relationship between BOP, GI and MPO activity, respectively. MPO activity is higher if BOP is present, and MPO activity is increasing with increase of GI score.

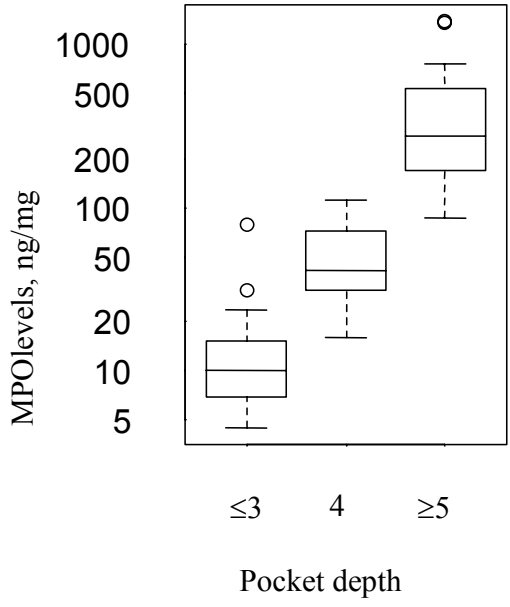

Figure 1. Probing pocket depth in association with total amounts of myeloperoxidase (MPO) in peri-implant sulcus fluid.

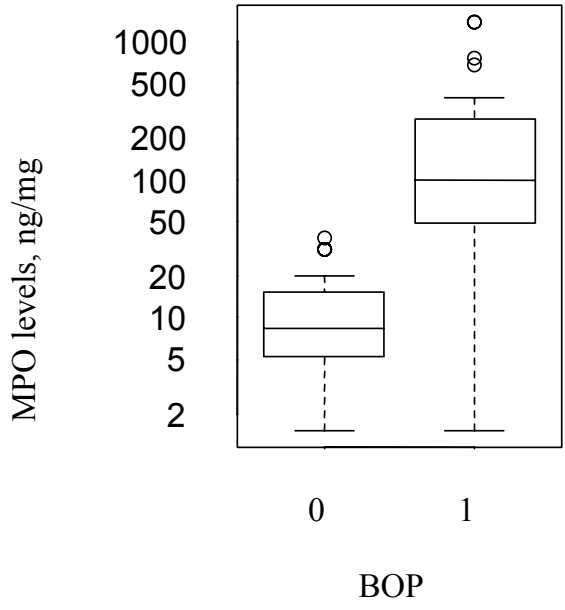

Figure 2. Bleeding on probing (BOP) in association with total amounts of myeloperoxidase (MPO) in peri-implant sulcus fluid.

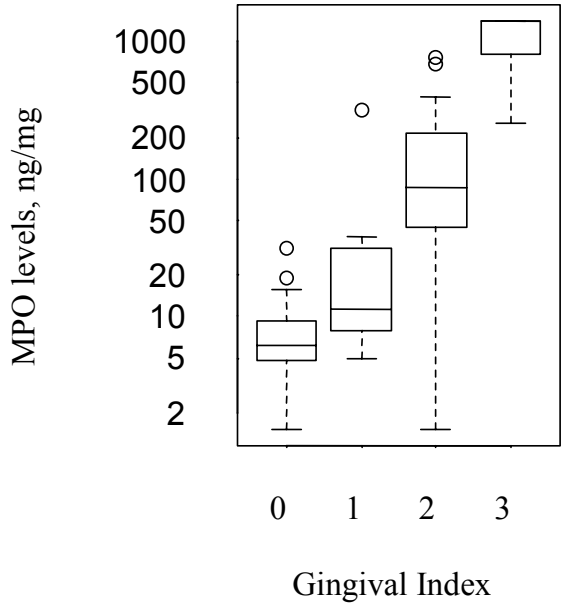

Figure 3. Gingival index in association with total amounts of myeloperoxidase (MPO) in peri-implant sulcus fluid.

5.3. Discussion

The present criteria for evaluating the disease status around dental implants are based on clinical and radiographic changes. Both criteria are often indicators of extensive pathologic changes and may not be reflective of current disease status. Radiographic examinations mainly reflect the past pattern of bone loss and may not reveal current disease status and phase of the actual disease activity, and, furthermore, the detectable radiographic bone loss reflects the peri-implantitis site that is already at the advanced and irreversible stage. Clinical parameters, including the gingival and plaque indices, are often subjective in nature (McClanahan et al. 2001) and their calibration for general clinical needs can be problematic. Measurements such as PPD and probing attachment levels are associated with examiner error and can be complicated by anatomic factors (Grossi et al. 1996). Moreover, the tissues surrounding dental implants have been found to be structurally different from the natural dentition. Around implants, the gingival tissues form a tight adhesion, similar to junctional epithelium around teeth. However, since no cementum is present, the periodontal probe can easily pass apically to the epithelial junction (Mombelli et al. 1997, Nishimura et al. 1997). As a result, the measurements for PPD may differ from those of the natural dentition, especially when inflammation is present. An often-used clinical parameter has been BOP. In healthy gingival tissue surrounding the implants, there is usually no detectible bleeding. However due to the differences in the attachment apparatus between teeth and dental implants, BOP may not be an accurate indicator of peri-implant inflammation. BOP has been reported to be a frequent finding around healthy dental implants (Berglundh et al.1991, Ericsson & Lindhe 1993).

The role of bacterial populations in peri-implant disease progression has also been investigated as a prognostic indicator (Rams et al. 1984, Mombelli et al. 1987). These investigations indicate trends similar to those found around the natural teeth. A shift from a gram-positive to a gram-negative population occurs with progression of disease. Other investigators have demonstrated that shallow pockets harbor non-pathologic bacterial populations, whereas deeper pockets (> 5 mm) are populated by a pathogenic microflora. However, the identification of specific bacterial species has not given clinicians the ability to determinate the presence of active disease or the risk of future disease progression.

The limitations associated with the use of traditional clinical parameters necessitate the development of objective biochemical markers to assess the peri-implant health. The early recognition of peri-implantitis would permit the clinician to intervene more rapidly, increasing treatment success. Recent investigations have attempted to characterize inflammatory mediators that may precede bone loss around dental implants. Interleukin-1β has been found to be elevated at diseased compared to healthy sites (Kao et al. 1993). Elevated levels of prostaglandin E_2 and platelet-derived growth factor have been associated

with risk of peri-implant disease progression (Salcetti et al. 1997). An early and objective means to monitor peri-implant health may be diagnostic MPO. This enzyme has been associated with inflammation of periodontal tissues (Wolff et al. 1988, Cao & Smith. 1989, Karhuvaara et al. 1990, Smith et al. 1992, Boutros et al. 1996, Yamalik et al. 2000).

During phagocytosis, some MPO is released from neutrophils and can react with H_2O_2 and Cl^- to form extracellular hypochlorous acid (HOCl). HOCl *in vitro* at low concentrations rapidly inactivates α_1-antiproteinase, the main inhibitor of serine proteases, such as elastase, in extracellular fluids. Elastase is present in the neutrophil azurophilic granules and, after being released, can hydrolyse connective tissue components such as elastin and collagen.

In the present cross-sectional study, two groups of implants were identified by clinical observations: a first group comprising healthy dental implants and a second group including fixtures with inflammatory lesions.

Because of the relatively small quantities of PISF, the level of MPO was reported as total activity per 1 mg sample, rather then concentration. This method of reporting biochemical parameters in GCF (and PISF) is appropriate in view of the findings of Smith et al. (1992), indicating that total amounts of GCF compounds per site are more closely associated with activity of periodontal disease than concentrations. One should also point out that saliva contamination of GCF might have a significant effect on the calculated concentration of an enzyme.

The study data indicated that elevated MPO levels were statistically associated with positive BOP index, GI ≥1 and PPD > 3 mm. These findings were similar to those of a previous study in which diseased and healthy sites of the natural dentition were compared with the MPO activity (Cao & Smith 1989). This investigation indicated that MPO levels are elevated at diseased sites, as compared to healthy sites within the same individuals. These results supported the concept that site-specific elevated MPO levels were associated with disease.

Only 9.4% of healthy implants had MPO levels higher than 25 $^{ng}/_{mg}$, at the same time in 96.9% of diseased implants MPO levels exceed 25 $^{ng}/_{mg}$ (Table 3).

Table 4 shows distribution of MPO levels using different levels of PPD. As can be seen, in all the implants with PPD ≥5 mm, the levels of MPO are higher than $25^{ng}/_{mg}$. 88.9 % of implants with PPD = 4 mm had MPO levels higher than 25 $^{ng}/_{mg.}$ Among the implants with PPD ≤3mm only in 10% of cases MPO levels exceeded $25^{ng}/_{mg.}$

Table 5 shows distribution of MPO levels using different GI scores. In implants with GI score 0, only 6.2% had MPO levels higher than 25 $^{ng}/_{mg}$, whereas all the implants with GI score 3 had MPO levels exceeding 25 $^{ng}/_{mg}$.

Table 6 shows distribution of MPO levels using different BOP index values. 15.6 % implants with no bleeding had MPO levels above 25 $^{ng}/_{mg}$; in implants where bleeding was present, this percent was 90.6.

Table 4. Distribution of myeloperoxidase (MPO) levels using different levels of probing pocket depth (PPD).

PPD, mm	MPO levels (geometric mean with 95% CI), ng/mg	% above 25 ng/mg
≤ 3	11.3 (8.2, 15.6)	10.0
4	44.0 (33.8, 57.4)	88.9
≥ 5	300.1 (189.7, 474.7)	100.0

Table 5. Distribution of myeloperoxidase (MPO) levels using different gingival index (GI) scores.

GI	MPO levels (geometric mean with 95% CI), ng/mg	% above 25 ng/mg
0	6.9 (4.8, 10.1)	6.2
1	15.6 (9.3, 26.2)	29.4
2	85.7 (52.6, 139.7)	89.3
3	785.8 (67.1, 9197.7)	100.0

Table 6. Distribution of myeloperoxidase (MPO) levels using different bleeding on probing (BOP) index values.

BOP	MPO levels (geometric mean with 95% CI), ng/mg	% above 25 ng/mg
0	9.4 (7.2, 12.3)	15.6
1	109.9 (66.8, 181.0)	90.6

Study data suggests that MPO level > 25 $^{ng}/_{mg}$ may be used to differentiate healthy and diseased implants.

The importance of these findings may be that in the absence of traditional parameters, such as BOP or gingival erythema, MPO may be a marker for disease progression at specific implants. An alternative hypothesis is that the presence of elevated MPO levels at specific implant sites, in conjunction with traditional signs of disease, may identify sites with an increased risk for peri-implant breakdown. It appears that the MPO test may be useful as an adjunct to traditional clinical parameters.

The results of this investigation should be interpreted with caution due to the cross-sectional design. We suggest that longitudinal monitoring of MPO in PISF may confirm its possible use as a marker and/or predictor of implant failure. It is worthy to add that such kind of studies are highly necessary because the elevation of MPO level has also an impact on development and progression of cardiovascular diseases (Pennathur et al. 2004, Ansell 2005).

6. PART III: CORRELATIONS BETWEEN CLINICAL PARAMETERS IND INTERLEUKIN-6 AND INTERLEUKIN-10 LEVELS IN SALIVA FROM TOTALLY EDENTULOUS PATIENTS WITH PERI-IMPLANT DISEASE

Recent evidence indicates that inflammatory cytokines, released by the host's monocytes and macrophages in response to bacterial products such as lipopolysaccharide and endotoxins, are significantly responsible for the breakdown processes of the periodontium in periodontitis. Recent advances in the understanding of biologic events involved in the pathogenesis of periodontitis indicate that bone-resorbing cytokine IL-6 (pro-inflammatory cytokine) may also be operative in the pathogenesis of peri-implant disease. In contrast, anti-inflammatory cytokine IL-10 may counteract peri-implant disease associated bone-resorption. With this background, the purpose of this study was to determine simultaneously the levels of IL-6 and IL-10 in peri-implant disease patients' saliva and to explore their relation with clinical parameters.

6.1. Patients and methods

6.1.1. Patient population

30 totally edentulous patients (62–70 years of age; 14 male and 16 female) with implant supported overdentures (Ankylos® dental implants, DentsplyFriadent, Germany) who were receiving maintenance care participated in this study. All the patients had two implants in lower jaw. The mean time in function was 67.2 ± 3.9 months (ranged between 61 and 72 months).

Prior to the start of the study the subjects gave their informed consent. Inclusion criteria were: (a) presence of 2 endosseous dental implants, (b) appropriate overdentures, (c) the same soft tissue biotype (keratinized soft tissue band present around dental implant) among all the patients, (d) healthy oral mucosa (no denture stomatitis etc), (e) no history of antibiotic treatment prior to the study for 3 months, (f) no history of systemic diseases that may affect cytokines levels in saliva, (g) no history of medical conditions that required antibiotic prophylaxis, (h) negative history of chronic corticosteroid use and (i) no history of medication that may alter the saliva secretion.

A complete oral examination was given prior to sialometry and measurement of clinical parameters.

6.1.2. Clinical examination

The clinical evaluations were performed by single examiner (S.L.) and included assessment of peri-implant PPD (mm), GI (0, 1, 2 or 3) (Löe & Silness 1963) and BOP (0 or 1). Clinical measurements were taken at four sites (mesial, buccal, distal and lingual). The PPD was measured to the nearest mm with a pressure-calibrated periodontal probe with a tip diameter of 0.5 mm and a probing force of 0.25 N (ClickProbe®, Hawe-Neos Dental, Bioggio, CH) (Karayannis et al. 1992, Lang et al. 1994).

6.1.3. Criteria for health and disease

Patients, who participated in the study, were placed into two different groups on the basis of PPD, GI and BOP as follows: patients with peri-implant disease (peri-implantitis and peri-implant mucositis) and healthy patients. The criteria have been referred to in previous reports (Pontoriero et al. 1994, Mombelli & Lang 1998). Briefly, peri-implantitis is defined as inflammation process around implants with loss of supporting bone and peri-implant mucositis is defined as reversible inflammation of the soft tissues with no loss of supporting bone.

The inclusion criteria were as follows: (1) in the healthy group both implants had no signs of peri-implant disease and in the peri-implant disease group both implants were affected with peri-implant disease (2) patients in healthy and diseased groups had to conform to the clinical parameters shown in Table 1.

Table 1. Clinical assessment and diagnosis of implants

Diagnosis	*PPD*	*GI*	*BOP*
Patients with peri-implant disease (n=12)	≥ 4 mm	≥ 1	1
Healthy patients (n=18)	≤ 3 mm	0	0

PPD – probing pocket depth, GI – Gingival Index, BOP – bleeding on probing

6.1.4. Sialometry

The whole unstimulated (resting) saliva specimens were obtained in the morning, and no oral stimulus was permitted for 90 min prior to collection. Five ml of whole saliva was collected by standardized gentle suction from the floor of the mouth into a sterile centifuge tube. The saliva was centrifuged for 5 min at 800 *g,* separated into 0.5-ml aliquots and frozen at –70° C until use.

All samples were assayed for blood contamination using an enzyme immunoassay kit for transferrin (Salimetrics). The latter is a large protein

presented in blood at very high concentrations. Its large size prevents transferrin from being passively or actively transported from the general circulation into saliva. Saliva samples were assayed for transferrin using an enzyme imonoassay as described by Kivlighan et al. (2004). Contaminated samples were not included in the study.

The total protein content was determined by the method of Lowry et al. (1951), using serum albumin as standard. Saliva from peri-implant disease group and healthy control group had protein concentrations of 2.91 ± 0.63 and 2.38 ± 0.51 mg/ml, respectively.

6.1.5. Cytokine assay

Assays for IL-6 and IL-10 were carried out according to manufacter's instructions (R&D Systems, Inc., Minneapolis, USA) and optical densities were measured at 450 nm using an ELISA reader. The lower limit of detection in the IL-6 assay was 0.7 pg/ml, and that in the IL-10 assay was 3.9 pg/ml. All samples were tested in duplicate, and the amount of cytokine present in each sample was determined by comparison with a standard curve constructed for each assay. The IL-6 and IL-10 contents were expressed as pg/ml in saliva. Previous studies confirmed that cytokines are stable in saliva (Leigh et al. 2001).

6.1.6. Statistical analysis

For categorical variables, number and percentages of patients in different categories and for continuous variables mean and standard deviation (SD) are presented. For statistical comparison between groups, Fisher's exact test was used for categorical variables and Wilcoxon Mann-Whitney test for continuous variables. All tests were conducted at conventional 5% significance level. For IL-6 and IL-10 levels, geometric mean with 95% confidence interval was calculated in subgroups defined by PPD, GI and BOP. These results were presented in the form of error bar graphs (Figures 1 to 3). For statistical analysis and graphs, software package R 2.0.0 for Windows (http://www.r-project.org) was used.

6.2. Results

6.2.1. Patient data and clinical results

12 patients showed signs of peri-implant disease. A second group of 18 patients with healthy peri-implant tissues was used as control.

Table 2 shows clinical status of each group including PPD, GI and BOP. Statistically significant differences for two groups were observed for all clinical parameters.

Table 2. Clinical data from healthy patients and patients with peri-implant disease

	Healthy (n=18)	***Diseased (n=12)***	***P***
PPD (mean ±SD)	2.63 (0.52)	3.85 (0.38)	0.003
% sites with			
gingival inflammation (GI >1)	0	57	0.026
bleeding on probing (BOP = 1)	0	100	0.0002

PPD – probing pocket depth, GI – Gingival Index, BOP – bleeding on probing

6.2.2. Biochemical analysis of saliva

Table 3 shows the total amounts of IL-6 and IL-10 in saliva in healthy and diseased groups. Significant differences were found between the two groups: p=0.001 and p=0.0006, respectively.

Table 3. Total amounts of IL-6 and IL-10 in saliva of healthy patients and patients with peri-implant disease, mean ± SD

	Healthy (n=18)	***Diseased (n=12)***	***P***
IL-6, pg/mg protein	0.30 (0.37)	3.33 (1.77)	0.001
IL-10, pg/mg protein	0.00 (0.00)	2.14 (0.74)	0.0006

Fig. 1 shows the relationship between PPD and concentration of cytokines. Concentration of IL-6 and IL-10 is increased with an increase in PPD. Figs 2 and 3 show relationship between BOP, GI and concentration of cytokines, respectively. Activity of IL-6 and IL-10 is higher if BOP is present, and activity of cytokines is increased with an increase in GI score.

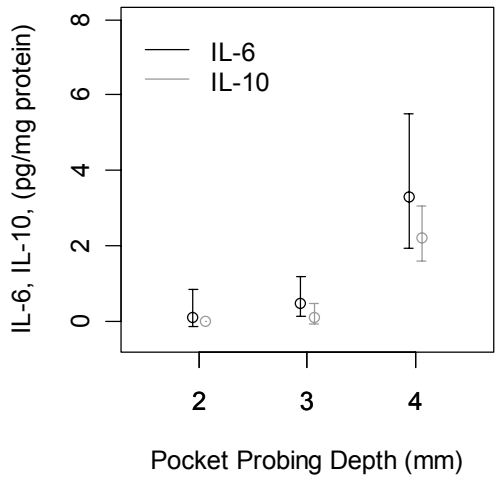

Figure 1. Geometric mean (95% CI) of total amounts of IL-6 and IL-10 in saliva in healthy and diseased patients in association with Pocket Probing Depth.

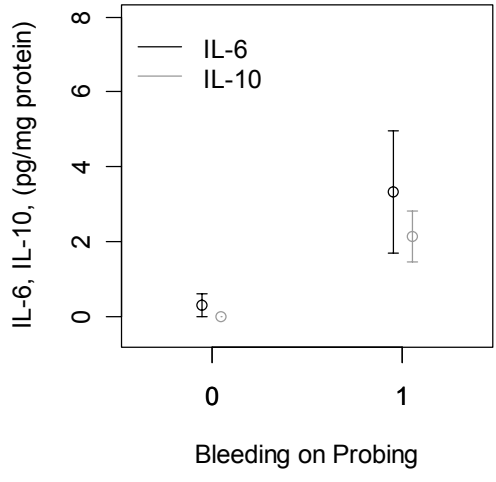

Figure 2. Geometric mean (95% CI) of total amounts of IL-6 and IL-10 in saliva in healthy and diseased patients in association with Bleeding on Probing.

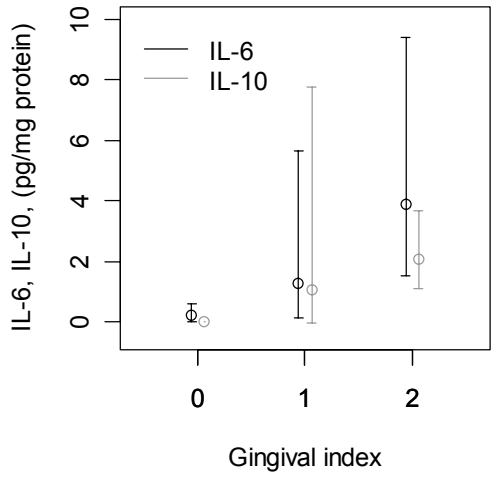

Figure 3. Geometric mean (95% CI) of total amounts of IL-6 and IL-10 in saliva in healthy and diseased patients in association with Gingival Index.

Table 4. Distribution of IL-6 and IL-10 levels using different levels of probing pocket depth (PPD), different gingival index scores (GI) and different bleeding on probing (BOP) index values

	PPD			***GI***			***BOP***	
	<3 mm	***3 mm***	***≥4 mm***	***0***	***1***	***≥2***	***0***	***1***
IL-6 levels, pg/mg protein, mean (SD)	0.11 (0.18)	0.62 (0.63)	3.61 (1.76)	0.31 (0.40)	1.61 (0.96)	4.27 (1.88)	0.30 (0.37)	3.33 (1.77)
% of patients with IL-6 levels above 1.5 pg/mg protein	0	17	100	0	75	100	0	100
IL-10 levels, pg/mg protein, mean (SD)	0.00 (0.00)	0.20 (0.50)	2.29 (0.68)	0.00 (0.00)	1.59 (1.30)	2.16 (0.72)	0.00 (0.00)	2.14 (0.74)
% of patients with IL-10 levels above 1.0 pg/mg protein	0	17	100	0	75	100	0	100

Table 4 shows the distribution of IL-6 and IL-10 levels using different levels of clinical parameters. In patients with PPD >3 mm, the levels of IL-6 and IL-10 are higher than 1.5 pg/mg protein and 1.0 pg/mg protein, respectively. In patients with GI score 0, IL-10 could not be detected, whereas the patients with GI score ≥2 had IL-6 and IL-10 levels exceeding 1.5 pg/mg protein and 1.0 pg/mg protein, respectively. In patients with no BOP present, IL-10 could not be detected. All the patients with positive BOP index had IL-6 and IL-10 levels exceeding 1.5 pg/mg protein and 1.0 pg/mg protein, respectively.

6.3. Discussion

The present study showed a significant difference in the levels of IL-6 and IL-10 in saliva between diseased patients and healthy controls. The large numbers of inflammatory cells in the connective tissues and connective tissue cells per se (i.e., fibroblasts and endothelial cells) can lead to the release of IL-6, stimulated by bacterial products and by interaction with the host cells (Dinarello 1989, Van-Snick 1990, Page 1991). Bartold et al. (1991) observed more intense IL-6 staining in the section of inflamed human gingiva than in the healthy gingival tissue. Furthermore, Matsuki et al. (1992) have indicated that there were prominent IL-6 mRNA-expressing cells in the inflamed gingival tissue. These reports support the fact that IL-6 levels are higher in diseased tissue than in healthy tissue as observed in the present study.

Some controversy exists in the scientific literature concerning the sources of cytokines in saliva. Recent evidence shows that there is no correlation between IL-6 levels in serum and saliva (Grisius et al. 1997, Sjögren et al. 2005). Findings in a recent study support the contention that salivary IL-6 is produced locally by acinar and/or ductal cells in the salivary glands (Boras et al. 2004). In addition, a difference in kinetics was also found for the IL-6 response in saliva and serum, indicating specific mechanisms for IL-6 production in saliva (Minetto et al. 2005). It is believed that saliva IL-6 is reflecting the response of the mucosal immune system (Sjögren et al. 2005). The study by Seymour et al. (2000) showed that inflammatory cytokines in whole saliva might be derived from gingival crevicular fluid (GCF). Increased levels of several cytokines such as IL-1, IL-2, IL-6, IL-8 and TNF-α have been observed in the GCF of patients with periodontal disease (Kamma et al. 2004). The penetration of bacteria and/or bacterial products into the tissues results in recruitment and activation of the monocyte/ T lymphocyte axis. This leads to the enhanced monocytic release of TNF-α, IL-1β and IL-6, associated with periodontal tissue destruction (Kamma et al. 2004). In conclusion, IL-6 is produced locally, by tissue cells in response to inflammatory stimulus and by cells in salivary glands reflecting the response of the mucosal immune system. Whatever the source of the salivary IL-6 and IL-10 is, it is possible to assess immunological patterns relevant to

systemic or local disease conditions (Rhodus et al. 1998, Tishler et al. 1999, Streckfus et al. 2001).

Certain levels of IL-6 could also be detected in patients with healthy peri-implant tissues. It is well-established fact that small numbers of macrophages and mononuclear cells are usually present in clinically healthy gingival tissues (Page & Schroeder 1976). All these cells and resident fibroblasts, endothelial cells, etc. could synthetize and release IL-6.

The results show that the level of IL-6 in peri-implant disease group is positively correlated with PPD, BOP and GI. These data suggest that a significant relationship exists between the amount of a pro-inflammatory IL-6 and the destruction of a peri-implant tissue. Thus the presence of elevated levels of IL-6 in saliva of patients with peri-implant disease along with the significant correlation with clinical assessments of peri-implant tissue destruction strongly suggests an important role for this mediator in the pathogenesis of peri-implant disease.

The results show that IL-10 could be detected only in saliva of patients with peri-implant disease and it did not appear at detectable levels in saliva of healthy controls. It is known that IL-10 inhibits bone resorption by suppressing cyclooxygenase-2-dependent prostaglandin E2 synthesis (Onoe et al. 1996, Allaedine et al. 1999). IL-10 also inhibits recruitment of osteoclast precursors and their differentiation to mature multinucleated osteoclasts (Xu et al. 1995, Owens et al. 1996). IL-10 response could be due to a specific Th1/Th2 response (Yamamoto et al. 1997). In patients with periodontal disease, activation of immune response by *Porphyromonas gingivalis* produces reactive T cells, which respond by secreting IL-10 (Gemmel et al. 1995, Gemmel et al 1997). IL-10 is a cytokine inhibiting factor that regulates the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α and INF-γ (Rossomando et al. 1990, Bartold & Haynes 1991, Reinhardt et al. 1993, Yamazaki et al. 1994). Therefore, the elevated levels of IL-10 in patients with peri-implant disease suggest that they play a certain role in the regulation of local immune response (Jinquian et al. 1992, Stein & Hendrix 1996). It was also reported that IL-10 was able to suppress excessive oxidative burst and its specific levels in saliva reflected also a counteraction of tissues to high-grade OxS events (Report of the American Academy of Periodontology 2002).

Scientific evidence shows that expression of IL-10 and some other cytokines is time-dependent. Schierano et al. (2003) assessed cytokines expression at different time-points after implant insertion. The results indicated that IL-10 expression increased at 4 month, decreased at 8 month and became almost undetectable at 12 month, suggesting that this cytokine combines to regulate immuno-inflammatory balance in peri-implant mucosa. The time factor was minimized in this study since all the patients had their endosseous implants in function for the period of 61 to 72 months (average time in function 67.2 ± 3.9 months).

The study data indicated that elevated levels of IL-6 and IL-10 were statistically associated with positive BOP index, GI ≥1 and PPD > 3 mm and were elevated in saliva of patients with peri-implant disease, as compared to healthy patients.

The data suggests that IL-6 levels > 1.5 pg/mg protein and IL-10 levels > 1.0 pg/mg protein may be used as the basis of a diagnostic test for peri-implant disease in patients with implant-supported overdentures. Advantages of using saliva for such diagnostic purposes include ease of sample collection, the large volume of fluid available for study and simple assay procedure.

Although the results of present study demonstrate increased production of IL-6 and IL-10 in patients with peri-implant disease, the relationship of these findings with disease progression remains to be established in the future.

7. PART IV: CHARACTERIZATION OF THE ANTIOXIDANT PROFILE OF HUMAN SALIVA IN PERI-IMPLANT HEALTH AND DISEASE

Peri-implant disease is considered to be an inflammatory disease, but many aspects of its pathogenesis remain unknown. At present, peri-implant disease is considered to be initiated and perpetuated by a small group of predominantly Gram-negative, anaerobic, or micro-aerophilic bacteria that colonize the subgingival area. Bacteria cause the observed tissue destruction directly by toxic products and indirectly by activating host defense systems, i.e. inflammation (Page & Kornman 1997). A variety of molecular species appear in the inflamed tissues, among them reactive species such as oxygen free radicals and other ROS.

The purpose of this study was to assess levels of various antioxidants and myeloperoxidase in resting and stimulated saliva and identify differences in their levels between the saliva of patients with healthy peri-implant tissues and patients with peri-implant disease.

7.1. Patients and methods

7.1.1. Patient population

Thirty totally edentulous patients (62–70 years of age; 14 male and 16 female) with implant supported overdentures (Ankylos® dental implants, DentsplyFriadent, Germany), from the group of patients who were receiving maintenance care participated in this study. All the patients had two implants in lower jaw. The mean time in function was 67.2 ± 3.9 months (ranged between 61 and 72 months).

Prior to the start of the study the subjects gave their informed consent. Inclusion criteria were: (1) presence of 2 endosseous dental implants, (2) appropriate overdentures, (3) the same soft tissue biotype (keratinized soft tissue band present around dental implant) among all the patients, (4) healthy oral mucosa (no denture stomatitis etc), (5) no history of antibiotic treatment prior to the study for 3 months, (6) no history of medical conditions that required antibiotic prophylaxis, (7) negative history of chronic corticosteroid use, (8) no history of medication interfering with saliva secretion and (9) no history of taking nutritional supplements for 3 months. Since smoking adversely affects antioxidant capacity in biological fluids (Zappacosta et al. 1999, Nagler et al. 2000, Reznick et al. 2003), all patients included in the study were non-smokers.

A complete oral examination was given prior to sialometry and measurement of clinical parameters.

7.1.2. Clinical examination

The clinical evaluations were performed by single examiner (S.L.) and included assessment of peri-implant PPD (mm), GI (0, 1, 2 or 3) (Löe et al. 1963) and BOP (0 or 1). The PPD was measured to the nearest mm with a pressure-calibrated periodontal probe with a tip diameter of 0.5 mm and a probing force of 0.25 N (ClickProbe®, Hawe-Neos Dental, Bioggio, CH) (Karayannis et al. 1992, Lang et al. 1994).

7.1.3. Criteria for health and disease

The included patients were classified on the basis of PPD, GI and BOP into two different groups as follows: patients with peri-implant disease (peri-implantitis and peri-implant mucositis) and healthy patients. The criteria have been referred to in previous reports (Pontoriero et al. 1994, Mombelli & Lang 1998). Briefly, peri-implantitis is defined as inflammation process around implants with loss of supporting bone and peri-implant mucositis is defined as reversible inflammation of the soft tissues with no loss of supporting bone.

The inclusion criteria were as follows: (1) in the healthy group both implants had no signs of peri-implant disease and in the peri-implant disease group both implants were affected with peri-implant disease (2) patients in healthy and diseased groups had to conform to the clinical parameters shown in Table 1.

Table 1. Clinical assessment and diagnosis of implants

Diagnosis	***PPD***	***GI***	***BOP***
Patients with peri-implant disease	≥ 4 mm	≥ 1	1
Healthy patients	≤ 3 mm	0	0

PPD – probing pocket depth, GI – Gingival Index, BOP – bleeding on probing

7.1.4. Sialometry

Unstimulated (resting) saliva specimens were obtained in the morning, and no oral stimulus was permitted for 90 min prior to collection. Saliva was collected before clinical evaluations were made. Whole saliva was collected by open suction method. Briefly, saliva was aspirated from the floor of the mouth into a test tube by a saliva ejector connected to the aspiration system. The ejector was

gently swept around the subject's mouth in a circular motion with one pass in the buccal vestibules and around and under the tongue at approximately 15-s intervals for 5 min. Subsequently, a 2% citric acid solution was applied to the tongue dorsum bilaterally at 30-s intervals. After 2 min, glands were again stimulated while whole saliva was collected by the same method. Salivary gland flow rates are expressed as volume of saliva (ml) secreted per minute. The mean volume of saliva secreted (ml/min) under resting conditions was 0.43 ± 0.17 ml/min. Under stimulated conditions this value was 1.00 ± 0.16 ml/min.

All samples were assayed for blood contamination using an enzyme immunoassay kit for transferrin (Salimetrics). The latter is a large protein presented in blood at very high concentrations. Its large size prevents transferrin from being passively or actively transported from the general circulation into saliva. Saliva samples were assayed for transferrin using an enzyme imonoassay as described by Kivlighan et al. (2004). Contaminated samples were not included in the study.

7.1.5. Sialochemical analysis

Myeloperoxidase activity in saliva

MPO levels were measured with Bioxytech® MPO Enzyme immonoassay (Oxis International, Portland, USA). The MPO-EIA assay system is a "sandwich" ELISA. Antigen captured by a solid phase monoclonal antibody is detected with a biotin-labeled goat polyclonal anti-MPO. An avidin alkaline phosphatase conjugate then binds to the biotinylated antibody. The alkaline phosphatase substrate p-nitrophenyl phosphate (pNPP) is added and the yellow product (p-nitrophenol) is monitored at 405 nm.

Salivary uric acid concentration

Uric acid concentration was measured with a kit supplied by Roche Diagnostics GmbH (Mannheim, Germany). In this assay, uric acid was transformed by uricase into allantoin and hydrogen peroxide, which under the catalytic influence of peroxidase oxidized the cromogen (4-aminophenazone/N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline) to form a red compound whose intensity of color was proportional to the amount of uric acid present in the sample.

Salivary ascorbic acid concentration

Ascorbic acid concentration was measured according to Ihara et al. (2000). Briefly, ascorbic acid is oxidized by 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy, free radical (TEMPO) to dehydroascorbic acid. The latter condenses with o-phenylenediamine (OPDA) to form a quinoxaline derivative that absorbs

light at 340 nm. The change in absorbance at 340 nm is proportional to the concentration of ascorbic acid in specimen.

Salivary total antioxidative status (TAS)

The assay used was based on a commercial kit supplied by Randox Laboratories (Ardmore, UK) in which metmyoglobin in the presence of iron was turned into ferrymyoglobin. Incubation of the latter with the Randox reagent 2,2´-azino-bis-[3-ethylbenzothiazoline sulfonate] (ABTS) resulted in the formation of a blue-green colored radical that could be detected at 600nm. Inhibition of the radical formation of $ABTS^{\bullet-}$ by antioxidant was calculated in millimolar quantities of ABTS disappearance.

7.1.6. Statistical analysis

For all variables mean and standard deviation for patients in different categories were presented. For statistical comparison between groups, Student's t-test was used. To archive approximate normal distribution, log-transformed values of MPO levels were used for testing. All tests were conducted at conventional 5 % significance level. For statistical analysis software package R 2.0.0 for Windows (http://www.r-project.org) was used.

7.2. Results

Patient data and clinical results

Twelve patients showed signs of peri-implant disease. A second group of 18 patients with healthy peri-implant tissues was used as control.

Table 2 shows patient demographics and clinical status of each group including PPD, gingival inflammation (GI) and BOP. Statistically significant differences for two groups were observed for all clinical parameters.

Table 2. Patient demographics (n=30) and clinical data

	Healthy (n=18)	***Diseased (n=12)***
Sex	8♂ and 10♀	6 ♂ and 6♀
Mean age (range)	66.0 ± 2.5 (62–70)	65.8 ± 2.2 (63–69)
PPD (mean ±SD)	2.63 (0.52)	3.85 (0.38)
% sites with		
gingival inflammation (GI ≥1)	0	100
bleeding on probing (BOP = 1)	0	100

BOP, bleeding on probing; PPD, probing pocket depth

Biochemical analysis of saliva

The total salivary antioxidant status is shown in *Table 3*, showing a significantly lower activity in patients with peri-implant disease as compared with healthy subjects. Significant differences were observed both in resting and stimulated saliva ($p=0.0004$ and $p<0.0001$, respectively). The results of measurements of individual antioxidants in healthy subjects are shown in *Table 4*. Calculation of antioxidant production rates (per minute) showed that when salivary flow is taken into account, significantly more urate ($p<0.0001$), ascorbic acid ($p<0.0001$) and myeloperoxidase ($p<0.0001$) is secreted during salivary stimulation. The antioxidant composition of the saliva from patients with peri-implant disease was measured and summarized in *Table 5*. We also observed that under stimulation, significantly more urate ($p<0.0001$), ascorbic acid ($p<0.0001$) and myeloperoxidase ($p<0.0001$) are secreted in saliva, if salivary flow rate is taken into account.

Table 3. Total Antioxidant Status (TAS) of resting (R) and stimulated (S) saliva samples from healthy and diseased subjects

	TAS (mM/l) ± s.d.	
	R	**S**
Healthy	0,41 ± 0,10	0,31 ± 0,09
Diseased	0,19 ± 0,07	0,12 ± 0,03
p	0.0004	<0.0001

Table 4. The myeloperoxidase (MPO), uric acid and ascorbate concentrations and production rates in resting (R) and stimulated (S) saliva samples from healthy subjects

Saliva type	**Uric acid (μM/l) mean ± s.d.**	**Uric acid (μM/min) mean ±s.d.**	**MPO (ng/ml) mean ± s.d.**	**MPO (ng/min) mean ±s.d.**	**Ascorbate (mg/l) mean ± s.d.**	**Ascorbate (mg/min) mean± s.d.**
R	307,20 ± 78,06	0,13 ± 0,03	15,03 ± 3,47	0,006 ± 0,001	2,79 ± 0,81	0,001 ± 0,001
S	241,50 ±89,09	0,24 ± 0,09	11,54 ± 1,46	0,012 ± 0,001	2,79 ± 0,81	0,003 ± 0,001

Table 5. The myeloperoxidase (MPO), uric acid and ascorbate concentrations and production rates in resting (R) and stimulated (S) saliva samples from subjects with peri-implant disease

Saliva type	Uric acid (μM/l) mean ± s.d.	Uric acid (μM/min) mean ± s.d.	MPO (ng/ml) mean ± s.d.	MPO (ng/min) mean ± s.d.	Ascorbate (mg/l) mean ± s.d.	Ascorbate (mg/min) mean ± s.d.
R	120,00 ±36,15	0,05 ±0,02	50,62 ± 18,23	0,022 ±0,008	1,54 ± 0,30	0,001 ±0,000
S	91,60 ± 39,35	0,09 ±0,04	26,16 ± 6,00	0,026 ±0,006	1,54 ± 0,30	0,002 ±0,000

Table 6 summarizes antioxidant status of saliva in healthy patients and patients with peri-implant disease. It was found that total antioxidant status of saliva in patients with peri-implant disease was significantly lower when compared to healthy controls. Concentrations of uric acid and ascorbate in saliva of healthy patients were significantly higher compared to patient with peri-implant disease. At the same time, myeloperoxidase activity in patients with peri-implant disease was significantly higher than that in healthy controls (*Table 7*).

Table 6. Antioxidant character of saliva from healthy subjects and subjects with peri-implant disease.

Antioxidants	Saliva					
	Resting			*Stimulated*		
	Healthy	*Diseased*	*p*	*Healthy*	*Diseased*	*p*
Urate, μM/l	307,2	120,0	<0.0001	241,5	91,60	0.0006
Ascorbate, mg/l	2,79	1,54	0.003	2,79	1,54	0.003
TAS, mM/l	0,41	0,19	0.0004	0,31	0,12	<0.0001

TAS, Total Antioxidant Status

Table 7. Myeloperoxidase (MPO) activity (ng/ml) of saliva from healthy subjects and subjects with peri-implant disease.

Saliva	MPO activity in saliva		
	Healthy	*Diseased*	*p*
Resting	15,0	50,6	0.001
Stimulated	11,5	26,2	0.0008

7.3. Discussion

Several studies indicated that there are increases in nitric oxide synthesis (Matejka et al. 1998), superoxide anion levels and myeloperoxidase activity (Cao & Smith 1989) in the inflamed periodontium. Reactive oxygen species (ROS) play important roles in physiological and immunoinflammatory reactions (Battino et al. 1999, Waddington et al. 2000). In the human body, there is an antioxidant mechanism to maintain the balance of oxidation-reduction (Teng 2003). The alterations of this balance (i.e. increased ROS) could lead to increased tissue damage directly by ROS. Several diseases have been correlated to an imbalance of oxidation-reduction or high-grade oxidative stress (Halliwell 1993, Liu & Mori 1999, Macnee et al. 1999, Zhang et al. 2000). There are two possible causes for the high-grade oxidative stress: increased ROS with no concomitant or less increased levels of antioxidants, or substantially decreased levels of antioxidants with no marked change of ROS. Enhanced superoxide anion production with no change of the antioxidant activity in gingival fluid of patients with chronic adult periodontitis, presumably due to the bacterial stimuli, has been reported (Guarnieri et al. 1991, Shapira et al. 1994). More recently, Brock et al. (2002) reported that antioxidant defense is reduced, and non-surgical therapy with improvements in clinical parameters can increase the antioxidant defense in chronic periodontitis patients. Similar results were reported in the recent study by Tsai et al. (2005).

The study of Sculley and Langley-Evans (2003) indicated that subjects with more severe periodontitis tended to have greater oxidative injury. This is wholly consistent with the hypothesis that there is enhanced ROS-mediated damage to tissues in the most advanced states of periodontal disease (Sculley & Langley-Evans 2002). It seems unlikely that oxidative processes play a dominating causal role in the aetiology of periodontitis, but they are likely to contribute to disease progression unless abated through antioxidant action. Recent study (Sculley & Langley-Evans 2003) has demonstrated that subjects with advanced periodontitis and evidence of oxidative injury had the lowest TAS rates. This is believed to be the consequence of antioxidant depletion due to the ongoing free radical activity and destruction of scavenging antioxidant species.

Recent evidence shows that urate is the major antioxidant component in saliva (Lenander-Lumikari et al. 1998, Sculley & Langley-Evans 2003). Urate, ascorbate and albumin contribute to the most of the antioxidant protection in whole saliva, but there is evidence of a salivary peroxidase enzyme too (Lenander-Lumikari et al. 1998). It is not clear whether the urate content of whole saliva directly reflects plasma concentrations or concentrations present in gingival crevicular fluid.

In the present study, two groups of patients were identified by clinical observations: a first group comprising patients with healthy dental implants and a second group including fixtures with inflammatory lesions. The current study

included a rigorous pre-sampling protocol. Since smoking is known as a source of ROS, "reducer" of antioxidants (Pryor & Stone 1993) and risk factor for periodontal disease (Haber et al. 1993), only non-smokers were included to the study. Patients having whether two healthy or two diseased implants were included to the healthy and diseased groups, respectively. Strict clinical criteria were used to identify healthy and diseased groups; patients were identified as diseased only when all clinical parameters were showing signs of disease (PPD $\geq$ 4 mm, GI $\geq$ 1 and BOP =1) and as healthy only when all clinical parameters allowed identifying peri-implant health (PPD $\leq$ 3 mm, GI =0 and BOP =0).

The study data indicated that MPO activity in saliva is increased in patients with peri-implant disease. These findings are similar to those of the previous study in which peri-implant sulcus fluid around diseased implants had higher MPO activity compared to healthy sites (Liskmann et al. 2004).

Total antioxidant status of saliva and concentration of uric acid and ascorbate, which are the main salivary antioxidants, are significantly decreased in patients with peri-implant disease. This may indicate that excessive ROS production in peri-implant disease leads to a high-grade oxidative stress, which may be an important factor contributing the destruction of peri-implant tissues.

Local decreases in antioxidant capacity in peri-implant sulcus fluid are likely to be of greater significance in the aetiology of peri-implant disease and associated damage to the implant supporting structures than the more systemic changes we have noted in whole saliva. The presence of antioxidants bathing the peri-implant sulcus may be of major importance in dampening down inflammatory processes initiated by bacterial infection. Obtaining and analyzing peri-implant sulcus fluid samples is, however, a complex process requiring a degree of specialism. The results of this study suggest that whole saliva may contain simply measured indicators of oxidative processes and may provide a useful tool for the development and monitoring of new treatment strategies. The importance of these findings may be also that the treatment of peri-implant disease may involve adjuvant anti-oxidants supplementation together with cumulative interceptive supportive therapy concept introduced by Mombelli & Lang (1998).

The results of this investigation should be interpreted with caution due to the cross-sectional design. We suggest that longitudinal monitoring of the antioxidant profile of saliva may confirm present results.

8. CONCLUSIONS

1. The study indicated the existence of high-grade cellular oxidative stress in patients with advanced periodontitis. An imbalance in the oxidant-antioxidant status may be one of the pathogenetic factors in the development of periodontal disease. Treatment with antioxidant vitamins and some minerals, lasting for 5 weeks, reduced inflammation and improved bleeding on probing index. This treatment variant enhanced cellular level of glutathione that diminished gingival inflammation.
2. Pathogenesis of peri-implant disease is associated with high-grade oxidative stress. Total antioxidant status of saliva and concentrations of the main salivary antioxidants, uric acid and ascorbic acid, are significantly decreased in patients with peri-implant disease. Concentration of MPO, an indicator of hyperfunctional PMNs, is also increased in patients with peri-implant disease. This indicates that excessive ROS production is associated with peri-implant disease leading to the situation of high-grade oxidative stress.
3. One of the possible markers of inflammation found in PISF is MPO, an enzyme originating from the granules of PMNs. The data indicate that elevated MPO levels are statistically associated with positive BOP index, GI ≥ 1, and PPD ≥ 3 mm. MPO levels are elevated at diseased sites, as compared to healthy sites within the same individuals. These results support the concept that site-specific MPO levels are associated with disease.
4. Possible markers of inflammation found in saliva are IL-6 and IL-10. Study data indicate that elevated levels of IL-6 and IL-10 are statistically associated with positive BOP index, GI ≥ 1, and PPD > 3 mm. IL-6 and IL-10 levels are elevated in saliva of patients with peri-implant disease as compared to healthy patients.
5. Only 9.4% of healthy implants had MPO levels higher than 25 $^{ng}/_{mg}$, while in 96.9% of diseased implants MPO levels exceed 25 $^{ng}/_{mg}$. Study data suggest that MPO level > 25 $^{ng}/_{mg}$ may be used to differentiate healthy and diseased implants.

   The importance of these findings may be that in the absence of traditional parameters, such as BOP or gingival erythema, MPO may be a marker for disease progression at specific implants. An alternative hypothesis is that the presence of elevated MPO levels at specific implant sites, in conjunction with traditional signs of disease, may identify sites with an increased risk for peri-implant breakdown. It appears that the MPO test may be useful as an adjunct to traditional clinical parameters.

   Study data suggest also that IL-6 levels > 3 pg/ml and IL-10 levels > 4 pg/ml may be used as the basis for a diagnostic test for peri-implant disease in patients with implant-supported overdentures. Advantages of using saliva for such diagnostic purposes include ease of sample collection, the large volume of fluid available for study and simple assay procedure.

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10. SUMMARY IN ESTONIAN
KOKKUVÕTE

PERI-IMPLANTSIUMI KAHJUSTUSED. PATOGENEES, DIAGNOOS JA RAVI: PÕLETIKU JA OKSÜDATIIVSE STRESSIGA SEOTUD ASPEKTID

Peri-implantsiumi kahjustused on põletikulised reaktsioonid, mis toimuvad implantaati ümbritsevates kudedes. Mukosiit on implantaati ümbritsevate pehmete kudede pöörduv põletik; peri-implantiidi tulemusena tekib püsiv implantaati ümbritseva luu destruktsioon.

Vähesed uuringud toovad välja andmeid peri-impantsiumi põletikuliste kahjustuste esinemissageduse kohta. Tavaliselt esitatakse marginaalse luu keskmine kadu kõigi uuritavate implantaatide osas, seetõttu andmed üksikute peri-implantsiumi põletikuliste kahjustustega implantaatide osas puuduvad. Sõltuvalt uuringust keskmiseks luukao määraks on 0.9 kuni 1.6 mm esimesel implantaadi funktsioneerimise aastal. Järgneva perioodi jooksul aastane luukao määr jääb vahemikku 0.05 kuni 0.13 mm.

Peri-implantsiumi põletikuliste kahjustuste etioloogia ja patogenees sarnaneb suurel määral parodontsiumi põletikuliste kahjustuste (gingiviidi ja parodontiidi) etioloogia ja patogeneesiga. Põletiku põhjustajaks on spetsiifilised parodontopatogeenid. Hambakatu akumuleerumine implantaadi pinnal põhjustab subepiteliaalse sidekoe infiltreerumist põletiku rakkudega ning epiteel haavandub. Kui hambakatt liigub apikaalsele, ilmneb radioloogiliselt diagnoositav koe destruktsioon nii hammaste, kui ka implantaatide ümber. Samas on peri-implantsiumi põletikulised kahjustused multifaktoriaalse geneesiga ning mõju avaldavad ka mitmed teised faktorid, nagu biomehhaaniline ülekoormus, traumaatilised kirurgilised meetodid, ebapiisav luu maht, häiritud peremeesorganismi vastus. Arvatakse, et ülemäärane reaktiivsete osakeste produktsioon on oluline faktor parodontiidi patogeneesis. Samas ei ole tehtud uuringuid, mis näitaksid ülemäärase oksüdatiivse stressi rolli peri-implantsiumi põletikuliste kahjustuste patogeneesis.

Peri-implantsiumi põlekuliste kahjustuste diagnostika põhineb hetkel radiograafiliste ja kliiniliste muutuste hindamisel implantaati ümbritsevates kudedes. Nad küll annavad informatsiooni peri-implantsiumi kahjustuse ulatusest, kuid ei peegelda haiguse hetkeseisu (aktiivne/inaktiivne) ega prognoosi haiguse progresseerumise riski. Seega traditsioonilised meetodid on prognostiliste ja diagnostiliste indikaatoritena piiratud. Samas oleks see äärmiselt vajalik peri-implantsiumi õigeks käsitlemiseks.

Käesoleva töö eesmärgid:

1) Hinnata antioksüdantide lisamanustamise raviefekti hammast või implantaati ümbritsevate kudede põletikuliste kahjustuste ravis.

2) Hinnata ülemäärase oksüdatiivse stressi võimalikku rolli peri-implantsiumi põletikuliste kahjustuste patogeneesis.
3) Määrata implantaaditasku vedeliku (PISF) biokeemiliste põletikumarkerite ja traditsiooniliste kliinilite parameetrite vahelist seost osalise hambutusega patsientidel, kellel esinesid peri-implantsiumi põletikulised kahjustused.
4) Määrata seost sülje biokeemiliste põletikumarkerite ja traditsiooniliste kliiniliste parameetrite vahel täieliku hambutusega katteproteesidega patsientidel, kellel esinesid peri-implantsiumi põletikulised kahjustused.
5) Hinnata biokeemiliste põletikumarkerite kasutamise võimalikkust peri-implantsiumi põletikuliste kahjustuste varajases diagnostikas.

I. Antioksüdantide lisamanustamise efekt parodontiidiga patsientidel

Uuriti 9 patsienti väljendunud parodontiidiga (uuritav rühm) ja 9 tervet patsienti (kontrollrühm). Patsientidel määrati traditsioonilisi kliinilisi parameetreid (sondeerimissügavus, veritsus sondeerimisel, hammaste liikuvus ning furkatsioonide haaratus) enne ja pärast antioksüdantide manustamist.

Patsientide süljes määrati oksüdatiivse stressi markereid. Uuriti taandatud (GSH) ja oksüdeeritud glutatiooni (GSSG) kontsentratsiooni, lipiidide peroksüdatsiooni markereid (TBARS ja LOOH), üldist antioksüdantset staatust (TAS) ja üldist antioksüdantset kaitset (TAP).

Antioksüdantide lisamanustamine toimus järgmise skeemi kohaselt: 60 mg koensüüm Q_{10} , 75 mg vitamiin E , 0.05 mg seleeni, 2.5 mg mangaani, 10 mg tsinki päevas, ning 100 mg α-lipoehapet igal teisel päeval.

Parodontiidi haigete igemeseisund paranes oluliselt 5 nädala möödudes peale raviskeemi rakendamist. Oluliselt vähenes ka veritsemine sondeerimisel. Olulised muutused olid ka biokeemiliste markerite osas. Glutatiooni redokssuhe (GSSG/GSH), mis on ülemäärase rakusisese oksüdatiivse stressi näitaja, vähenes oluliselt (2.30 ja 0.46, vastavalt enne ja peale ravi). LOOH tase oli oluliselt kõrgem parodontiidi haigetel võrreldes tervete kontrollidega ning LOOH tase normaliseerus peale ravi.

Uuringu tulemused näitasid, et väljendunud parodontiidiga patsientidel on oluliselt tõusnud rakusisese oksüdatiivse stressi tase. Tundub, et tasakaalu puudumine oksüdantide-antioksüdantide vahel võib olla üheks oluliseks faktoriks parodontiidi patogeneesis. Raviskeem antioksüdantide manustamisega 5 nädala jooksul viis põletiku taandarenemisele: kliiniliselt vähenes veritsus, paranes kudede turgor ja pikenes remissiooni faas. Selline ravi variant suurendas rakusiseselt glutatiooni taset ning vähendas põletikunähte.

II. Peri-implantsiumi kliiniline seisund ja müeloperoksüdaasi tase implantaaditaskuvedelikus

Uuriti 65 implantaati 24 patsientil. Kolmekümne neljal implantaadil esinesid peri-implantsiumi põletikulised kahjustused. Teine grupp (31 implantaati) terve peri-implantsiumiga oli kontrollrühmaks.

Määrati traditsioonilisi kliinilisi parameetreid (tasku sondeerimissügavus, veritsuse esinemine sondeerimisel, gingiviidi indeks) ning sooritati implantaaditaskuvedeliku kogumine mikropipeteerimise teel. Müeloperoksüdaasi sisaldust implantaaditasku vedelikus määrati immunoensümaatilisel meetodil (ELISA).

Uuringutulemused näitasid, et kõrgenenud MPO tase on statistiliselt seotud veritsuse esinemisega sondeerimisel, igemeindeksiga ≥1 ja sondeerimissügavusega > 3 mm. Uuring näitas, et MPO tase on suurenenud haigete implantaatide taskuvedelikus võrreldes tervete implantaatidega samal indiviidil. Ainult 9.4% tervetel implantaatidel MPO tase oli kõrgem kui 25 $^{ng}/_{mg}$, samas 96.9% haigetel implantaatidel MPO tase ületas 25 $^{ng}/_{mg}$. Saadud andmete põhjal võib järeldada, et MPO taset >25 $^{ng}/_{mg}$ võib kasutada tervete ja haigete implantaatide eristamiseks.

III. Seosed kliiniliste parameetrite ja interleukiin-6 ning interleukiin-10 taseme vahel täielikult hambutute patsientide süljes, kellel esinesid peri-implantsiumi põletikulised kahjustused

Uuriti 30 hambutut patsienti implantaatidele toetuvate katteproteesidega. Kõikidel patsientidel oli 2 implantaati alalõuas.

Määrati traditsioonilisi kliinilisi parameetreid (tasku sondeerimissgavus, veritsuse esinemine sondeerimisel, igeme indeks). Kliiniliste parameetrite alusel jaotati patsiente kahte rühma – a) terved ja b) patsiendid peri-implantsiumi põletikuliste kahjustustega. Tsütokiinide sisaldust süljes määrati immunoensümaatilisel meetodil (ELISA).

Uuringu tulemused näitasid, et IL-6 tase süljes on oluliselt kõrgenenud peri-implantsiumi põletikuliste kahjustustega patsinetidel. IL-10 oli võimalik määrata ainult peri-implantsiumi põletikuliste kahjustustega patsientidel. Lisaks sellele, IL-6 ja IL-10 tase haigete patsientide rühmas positiivselt korreleerus kliiniliste parameetritega.

Saadud andmete põhjal võib järeldada, et põletiku soodustava tsütokiini (IL-6) taseme ja peri-implantsiumi destruktsiooni vahel esineb oluline seos. Tulemused lubavad eeldada, et IL-6 ja IL-10 taseme määramist süljes sõib kasutada peri-implantsiumi põletiku markeritena.

IV. Sülje antioksüdantse profiili iseloomustus terve ja põletikulise peri-implantsiumiga patsientidel

Uuriti 30 hambutut patsienti implantaatidele toetuvate katteproteesidega. Kõikidel patsientidel oli 2 implantaati alalõuas. Patsientidel olid määratud järgmised biokeemilised ja kliinilised parameetrid: kusihape, askorbiinhape ja müeloperoksüdaasi sisaldus süljes, sülje totaalne antioksüdantne staatus, tasku sondeerimissügavus, igeme indeks ja veritsus sondeerimisel.

Sülje üldine antioksüdantne staatus (TAS) ja sülje peamiste antioksüdantide – kusihappe ning askorbiinhappe – sisaldus on oluliselt vähenenud põletikulise peri-implantsiumiga patsientidel. Sellised tulemused näitavad, et ülemäärane hapniku reaktiivsete osakeste tootmine põletikulises peri-implantsiumis põhjustab ülemäärast oksüdatiivset stressi, mis võib olla oluliseks faktoriks, mis soodustab implantaati ümbritsevate kudede destruktsiooni.

Kirjeldatud andmete olulisus seisneb selles, et nad aitavad paremini mõista peri-implantsiumi põletikuliste haiguste patogeneesi. Samuti võib nimetatud haiguste ravi sisaldada peale Mombelli ja Lang'i (1998) poolt tutvustatud raviprotokolli (CIST) ka antioksüdantide manustamist.

11. ACKNOWLEDGEMENTS

I wish to express my warm and sincere gratitude to all those who made this work possible, with special attention to:

Professor **Mihkel Zilmer**, my principal supervisor, for giving me the opportunity to do this work, for his constant support, constructive criticism, excellent teaching not only in the field of biochemistry but also in "life sciences";

Assistant professor **Tiiu Vihalemm**, my co-supervisor, for introducing me to the interesting field of biochemistry, for always being supportive and genuinely interested in my work, for always having her door open and taking time whenever needed;

Assistant professor **Olev Salum**, my co-supervisor, for introducing me to the fascinating field of dental prosthodontics and implantology, for all his important advices, for always being kind, patient and supportive;

Assistant professor **Krista Fischer**, for her valuable statistical advice;

Mrs.**Tiiu Kullisaar** and Mrs.**Kersti Zilmer**, for the generous help and supervision in the lab;

Assistant professor **Mare Saag**, for the invaluable help in collecting the clinical data and the competent revision of the thesis;

Professor emeritus **Rein Teesalu**, for the competent revision of the thesis;

Dr. **Valdur Viikmann**, for introducing me to the field of dental implantology, for providing excellent working conditions in the dental clinic, for his understanding and support;

Ms. **Evely Suija**, for her valuable technical assistance;

My beloved **parents**, for their unconditional love and support and for always being with me when I needed them;

My warmest thanks and love goes to **Maria** for supporting me throughout good and bad times and especially to my son **Simon** for just being a wonderful son.

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CORRELATIONS BETWEEN CLINICAL PARAMETERS AND INTERLEUKIN-6 AND INTERLEUKIN-10 LEVELS IN SALIVA FROM TOTALLY EDENTULOUS PATIENTS WITH PERI-IMPLANT DISEASE

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ABSTRACT

Liskmann, S., Vihalemm, T., Salum, O., Zilmer, K., Fischer, K. and Zilmer,M. Correlations between clinical parameters and Interleukin-6 and Interleukin-10 levels in saliva from totally edentulous patients with peri-implant disease.

Purpose
The purpose of the present study was (1) to assess the relation between clinical parameters and concentration of the pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 in saliva of totally edentulous patients with implant-supported overdentures; (2) to assess whether estimation of IL-6 and IL-10 levels in saliva could be a useful laboratory tool to detect changes preceding serious clinical complications.

Materials and Methods
30 healthy adult volunteers (14 men and 16 women) with implant-supported overdentures were recruited from Tallinn Dental Clinic. Biochemical and clinical parameters evaluated were the following ones: the levels of IL-6 and IL-10 in saliva, pocket probing depth (mm), Gingival Index (0,1,2 or 3), and bleeding on probing (0 or 1).

Results
Our results showed that the level of IL-6 in saliva in peri-implant disease group was significantly elevated compared to healthy group. IL-10 could be detected only in saliva of patients with peri-implant disease and it did not appear at detectable amounts in saliva of healthy controls. In addition, the levels of IL-6 and IL-10 in peri-implant disease group were positively correlated with clinical parameters.

Conclusions
These data suggest that a significant relationship exists between the amount of a pro-inflammatory cytokine (IL-6) and the inflammatory response in peri-implant tissue. Our results suggest also that IL-6 and IL-10 could be used as markers of peri-implant disease together and the level of the latter cytokine gives additional information how potent is really an integrated immune response of organism for maintenance of inflammatory balance.

KEY WORDS

IL-6, IL-10, dental implants, saliva, peri-implantitis, peri-implant disease.

INTRODUCTION

The long term success of osseointegrated implants as replacements for missing teeth relies, among other factors, on maintaining the integrity of the biological seal of the peri-implant tissues (1). The peri-implant sulcus is, anatomically, functionally and environmentally quite similar to the periodontal crevice (2, 3),

and provides a niche for the colonization and growth of oral microorganisms. When the host response to plaque accumulation results in reversible soft tissue reactions, it is termed as peri-implant mucositis, whereas term peri-implantitis is used to describe irreversible inflammatory reactions with the loss of supporting bone in the tissues surrounding a functioning implant (4). Animal and human studies have shown that peri-implant infection appears to have similar clinical, radiographic and histologic features to periodontitis (5, 6, 7, 8).

Recent evidence indicates that inflammatory cytokines, released by the host's cells in response to bacterial products such as lipopolysaccharide and endotoxins, are substantially responsible for the breakdown processes of the periodontium in periodontitis (9, 10).

An important aspect of the host response is the cell-mediated immunity of which T cells are the main components. The majority of T cells express the T cell receptor that recognizes processed antigens in conjunction with major histocompatibility complex molecules. These cells release a variety of cytokines following antigen recognition. Two subsets of CD4+ T helper (Th) cells have been defined based on the pattern of cytokine secretion. Cells of the Th1 subset secrete interleukin (IL)-2, interferon (INF)-γ and INF-α while the Th2 cell subset secretes IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (11). It has to be mentioned that the synthesis of IL-2, IL-6, IL-10 and IL-13 is not tightly restricted to a single subset (11).

Although cytokines are produced by locally infiltrated immunocompetent cells such as T-cells and monocytes at the diseases sites, cell types that normally compose the tissue – such as fibroblasts, epithelial cells and endothelial cells – are also involved in cytokine production during the inflammatory response (11).

An inflammatory cytokine is defined as a cytokine that is induced during the course of an inflammatory response and is closely associated with its onset and/or progression. Thus far, IL-1α, IL-1β, IL-6, IL-8 and TNF-α are generally classified as inflammatory cytokines (11). Since one prominent feature of periodontal disease is resorption of alveolar bone, particular attention has been paid to the roles of IL-1α, IL-1β, IL-6, IL-8 and TNF-α in the pathogenesis process, due to their enhancement of bone resorption (11).

Some studies (12, 13) have previously shown that interleukin-1β (IL-1β) is present at elevated levels in the gingival crevicular fluid (GCF) and in tissue from periodontal pockets. This cytokine stimulates bone resorption, prostaglandin synthesis and protease production by many cell types including fibroblasts and osteoblasts (14, 15, 16, 17, 18). Interleukin-6 (IL-6) is a proinflammatory cytokine and central mediator of the acute-phase response. This pleiotropic cytokine stimulates B cell differentiation and T cell activation as well as hepatocyte production of acute phase proteins, including CRP (19). In addition, it can induce bone resorption, by activating cells to release secondary mediators, which are responsible for tissue destruction. (20, 21). Spontaneous production

of IL-6 has been reported in mononuclear cells isolated from inflamed gingival tissues of patients with periodontitis (22). The presence of IL-6 bearing cells in inflamed gingival tissue also has been demonstrated by other researchers (23, 24). In a longitudinal study, IL-6 content in GCF was found to be correlated with the severity of periodontal disease (25).

Interleukin-10, a cytokine with potent anti-inflammatory properties, has been implicated in the regulation of both both cellular and humoral immune responces (26, 27, 28, 29). Studies indicate that lack of IL-10 can render a host susceptible to a bacteria –initiated chronic inflammatory condition (30). IL-10 has a major role in regulating pro-inflammatory cytokine levels *in vivo*, e.g. IL-1 and tumor necrosis factor production in response to various inflammatory stimuli is elevated in the absence of IL-10 or curtailed by IL-10 administration (28, 31, 32). Also, IL-10 have been reported to down-regulate the production of both IL-1 (33, 34, 35, 36, 37) and the IL-6 (38, 39). Sasaki et al. (40) found that IL-10 supresses infection-stimulated bone resorption in vivo.

Although extensive research has been done in the area of periodontal inflammatory mediators, few have studied the role of the host immune response in peri-implant disease. By our knowledge there are no studies regarding the role of IL-6 and IL-10 in the pathogenesis of peri-implant disease at present time.

Recent advances in the understanding of biologic events involved in the pathogenesis of periodontitis indicate that pro-infammatory cytokine IL-6 may also be operative in the pathogenesis of peri-implant disease. On the contrary, anti-inflammatory cytokine IL-10 may suppress peri-implant disease associated tissue destruction.

Since all the patients participating in the study had no natural teeth (upper full denture and lower implant-supported overdenture wearers), we choose whole saliva as a medium for diagnostics. The overall advantages of using saliva as a diagnostic fluid have been recently reviewed (41). The advantaged of saliva compared to peri-implant sulcus fluid are (a) simple collection procedure and (b) non-invasive method of collection. Both issues were especially important concerning the age of patients (varied between 62 and 70 years). Quantification of cytokines in saliva have been made in several pathologic conditions, i.e. in patients with denture stomatitis (42), in HIV-positive smokers with oropharyngeal candidasis (43), in oral lichen planus patients (44).

The purpose of this study was (a) to determine simultaneously the levels of IL-6 and IL-10 in peri-implant disease patients' saliva and to explore their relation with clinical parameters; (b) to assess whether estimation of IL-6 and IL-10 levels in saliva could be a useful laboratory tool to detect changes preceding serious clinical complications.

MATERIALS AND METHODS

Patient population

30 totally edentulous patients (14 men, 16 women) with implant supported overdentures (Ankylos® dental implants, DentsplyFriadent, Germany) who were receiving maintenance care participated in this study. All the patients had two implants in the mandible. The mean time in function was 67.2 ± 3.9 months (varied between 61 and 72 months).

Prior to the start of the study the subjects gave their informed consent. Inclusion criteria were: (a) presence of 2 endosseous dental implants, (b) appropriate overdentures, (c) the same soft tissue biotype among all the patients, (d) healthy oral mucosa (no denture stomatitis etc), (e) no history of antibiotic treatment prior to the study for 3 months, (f) negative history of systemic diseases affecting cytokines levels in saliva, (g) no history of medical conditions that required antibiotic prophylaxis, (h) negative history of chronic corticosteroid use and (i) no history of medication interfering with saliva secretion.

A complete oral examination was given prior to sialometry and measurement of clinical parameters.

Clinical examination

The clinical evaluations were performed by single examiner (S.L.). The clinical examinations included assessment of peri-implant pocket probing depth (PPD) (mm), Gingival Index (GI) (0, 1, 2 or 3) (45) and bleeding on probing (BOP) (0 or 1). Clinical measurements were taken at four sites (mesial, buccal, distal and lingual). The pocket probing depth was measured to the nearest mm with a pressure-calibrated periodontal probe with a tip diameter of 0.5 mm and a probing force of 0.25 N (ClickProbe®, Hawe-Neos Dental, Bioggio, CH) (46, 47).

Criteria for health and disease

Studied patients were categorized on the basis of pocket probing depth (PPD), gingival index (GI) and bleeding on probing (BOP) into two different groups as follows: patients with peri-implant disease (peri-implantitis and peri-implant mucositis) and healthy patients. The criteria have been referred to in previous reports (48, 49). Briefly, peri-implantitis is defined as inflammation process around implants with loss of supporting bone and peri-implant mucositis is defined as reversible inflammation of the soft tissues with no loss of supporting bone. According to these definitions we applied the clinical parameters as shown in Table 1. If one of the implants was healthy and the other showed signs of peri-implant disease, a patient was categorized as diseased.

Table 1. Clinical assessment and diagnosis of implants

Diagnosis	*PPD*	*GI*	*BOP*
Patients with peri-implant disease (n=12)	≥ 4 mm	≥ 1	1
Healthy patients (n=18)	≤ 3 mm	0	0

BOP, bleeding on probing; GI, gingival index; PPD, pocket probing depth

Saliva collection

The whole unstimulated (resting) saliva specimens were obtained in the morning, and no oral stimulus was permitted for 90 min prior to collection. 5 ml of whole saliva was collected by standardized gentle suction from the floor of the mouth into a sterile centifuge tube. The saliva was centrifuged for 5 min at 800 *g,* separated into 0.5-ml aliquots and frozen at –70° C until use.

All samples were assayed for blood contamination using an enzyme immunoassay kit for transferrin (Salimetrics). Transferrin is a large protein prevalent in blood at very high concentrations. Its large size prevents transferrin from being passively or actively transported from the general circulation into saliva. Saliva samples were assayed for transferrin using an enzyme imonoassay as described by Kivlighan et al. 2004 (50). Contaminated samples were not included in the study.

The total protein content was determined by the method of Lowry et al. (51), using serum albumin as standard. Saliva from peri-implant disease group and healthy control group had protein concentrations of 2.91 ± 0.63 and 2.38 ± 0.51 mg/ml, respectively.

Cytokine assay

The amounts of IL-6 and IL-10 were determined by using commercially available enzyme-linked immunoadsorbent assays (ELISAs) (R&D Systems, Inc., Minneapolis, USA), specific for each cytokine. The assays were sandwiched and performed according to the manufacturer's instructions using human recombinant standards. All samples were tested in duplicate. The IL-6 and IL-10 contents were expressed as pg/mg protein. Previous studies confirmed that cytokines are stable in saliva (52).

Statistical analysis

For categorical variables, number and percentages of patients in different categories and for continuous variables mean and standard deviation (SD) are presented. For statistical comparison between groups, Fisher's exact test was used for categorical variables and Wilcoxon Mann-Whitney test for continuous variables. All tests were conducted at conventional 5% significance level. For IL-6 and IL-10 levels geometric mean with 95% confidence interval was calculated in subgroups defined by Pocket Probing Depth, Bleeding on Probing

and Gingival Index. These results were presented in the form of error bar graphs (Figures 1 to 3). For statistical analysis and graphs, software package R 2.0.0 for Windows (http://www.r-project.org) was used.

RESULTS

Patient data and clinical results

12 patients showed signs of peri-implant disease. A second group of 18 patients with healthy peri-implant tissues was used as control.

Table 2 shows clinical status of each group including PPD, gingival inflammation (GI) and BOP. Statistically significant differences for two groups were observed for all clinical parameters.

Table 2. Clinical data from healthy patients and patients with peri-implant disease

	Healthy (n=18)	***Diseased (n=12)***	***P***
PPD (mean ±SD)	2.63 (0.52)	3.85 (0.38)	0.003
% sites with			
gingival inflammation (GI >1)	0	57	0.026
bleeding on probing (BOP = 1)	0	100	0.0002

BOP, bleeding on probing; PPD, probing pocket depth

Biochemical analysis of saliva

Table 3 shows the total amounts of IL-6 and IL-10 in saliva in healthy and diseased groups. Significant differences were found between the two groups: p=0.001 and p=0.0006, respectively.

Table 3. Total amounts of IL-6 and IL-10 in saliva of healthy patients and patients with peri-implant disease, mean ± SD

	Healthy (n=18)	***Diseased (n=12)***	***P***
IL-6, pg/mg protein	0.30 (0.37)	3.33 (1.77)	0.001
IL-10, pg/mg protein	0.00 (0.00)	2.14 (0.74)	0.0006

Clinical parameters and levels of cytokines

Fig. 1 shows the relationship between PPD and concentration of cytokines. As can be seen, concentration of IL-6 and IL-10 is increasing with the increase of PPD. Figs 2 and 3 show relationship between BOP, GI and concentration of cytokines, respectively. Activity of IL-6 and IL-10 is higher if BOP is present, and activity of cytokines is increasing with increase of GI score.

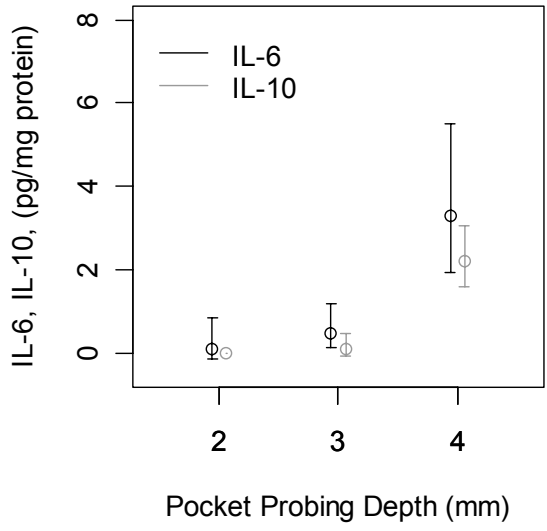

Figure 1. Geometric mean (95% CI) of total amounts of IL-6 and IL-10 in saliva in healthy and diseased patients in association with Pocket Probing Depth.

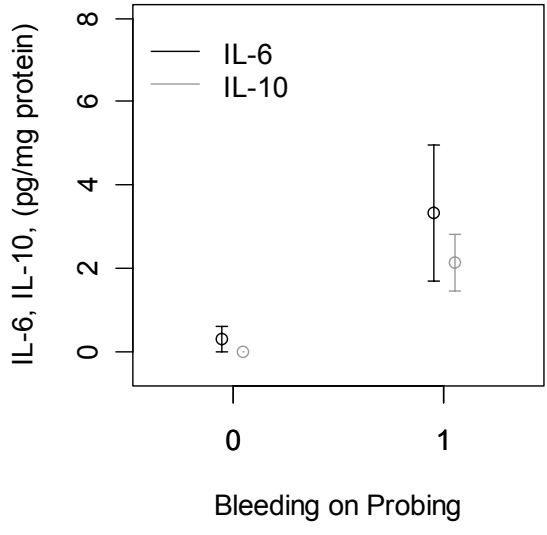

Figure 2. Geometric mean (95% CI) of total amounts of IL-6 and IL-10 in saliva in healthy and diseased patients in association with Bleeding on Probing.

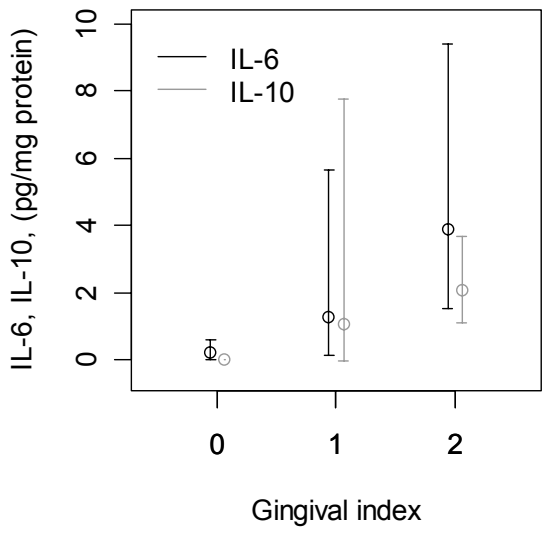

Figure 3. Geometric mean (95% CI) of total amounts of IL-6 and IL-10 in saliva in healthy and diseased patients in association with Gingival Index.

Table 4 shows the distribution of IL-6 and IL-10 levels using different levels of clinical parameters. As can be seen, in all patients with PPD >3 mm, the levels of IL-6 and IL-10 are higher than 1.5 pg/mg protein and 1.0 pg/mg protein,

Table 4. Distribution of IL-6 and IL-10 levels using different levels of pocket probing depth (PPD), different gingival index scores (GI) and different bleeding on probing (BOP) values

	PPD			***GI***			***BOP***	
	< 3 mm	***3 mm***	***≥ 4 mm***	***0***	***1***	***≥2***	***0***	***1***
IL-6 levels, pg/mg protein, mean (SD)	0.11 (0.18)	0.62 (0.63)	3.61 (1.76)	0.31 (0.40)	1.61 (0.96)	4.27 (1.88)	0.30 (0.37)	3.33 (1.77)
% of patients with IL-6 levels above 1.5 pg/mg protein	0	17	100	0	75	100	0	100
IL-10 levels, pg/mg protein, mean (SD)	0.00 (0.00)	0.20 (0.50)	2.29 (0.68)	0.00 (0.00)	1.59 (1.30)	2.16 (0.72)	0.00 (0.00)	2.14 (0.74)
% of patients with IL-10 levels above 1.0 pg/mg protein	0	17	100	0	75	100	0	100

respectively. In patients with GI score 0, IL-10 could not be detected, whereas all the patients with GI score ≥2 had IL-6 and IL-10 levels exceeding 1.5 pg/mg protein and 1.0 pg/mg protein, respectively. In patients with no bleeding present, IL-10 could not be detected. All the patients with bleeding on probing had IL-6 and IL-10 levels exceeding 1.5 pg/mg protein and 1.0 pg/mg protein, respectively.

DISCUSSION

The present study showed a significant difference in the levels of IL-6 and IL-10 in saliva between diseased patients and healthy controls. The large numbers of inflammatory cells in the connective tissues and connective tissue cells per se (i.e., fibroblasts and endothelial cells) can lead to the release of IL-6, stimulated by bacterial products and by interaction with the host cells (53, 20, 19). Bartold et al. (23) observed more intense IL-6 staining in the section of inflamed human gingiva than in the healthy gingival tissue. Furthermore, Matsuki et al. (24) have indicated that there were prominent IL-6 mRNA-expressing cells in the inflamed gingival tissue. These reports support the fact that IL-6 levels are higher in diseased tissue than in healthy tissue as observed in the present study.

Some controversy exists in the scientific literature concerning the sources of cytokines in saliva. Recent evidence shows that there is no correlation between IL-6 levels in serum and saliva (54, 55). Findings in a recent study support the contention that salivary IL-6 is produced locally by acinar and/or ductal cells in the salivary glands (56). In addition, a difference in kinetics was also found for the IL-6 response in saliva and serum, indicating specific mechanisms for IL-6 production in saliva (57). It is believed that saliva IL-6 is reflecting the response of the mucosal immune system (55). Study of R.A.Seymour et al. (58) shows that inflammatory cytokines in whole saliva might be derived from gingival crevicular fluid (GCF). Increased levels of several cytokines such as IL-1, IL-2, IL-6, IL-8 and TNF-α have been observed in the GCF of patients with periodontal disease (59). The penetration of bacteria and/or bacterial products into the tissues results in recruitment and activation of the monocyte/ T lymphocyte axis. This leads in turn to the enhanced monocytic release of TNF-α, IL-1β and IL-6, associated with periodontal tissue destruction (59). In conclusion one may say that IL-6 is produced locally, by tissue cells in response to inflammatory stimulus and by cells in salivary glands reflecting the response of the mucosal immune system. Despite the source of the salivary IL-6 and IL-10, it is suitable to assess immunological patterns relevant to systemic or local disease conditions (60, 61, 62).

It is normal that certain levels of IL-6 could also be detected in patients with healthy peri-implant tissues. It is well established fact that small numbers of

macrophages and mononuclear cells are usually present in clinically healthy gingival tissues (63). All these cells and resident fibroblasts, endothelial cells, etc. could synthetize and release IL-6.

The results show that the level of IL-6 in peri-implant disease group is positively correlated with PPD, BOP and GI. These data suggest that a significant relationship exists between the amount of a pro-inflammatory (IL-6) and the destruction of a peri-implant tissue. Thus the presence of elevated levels of IL-6 in saliva of patients with peri-implant disease along with the significant correlation with clinical assessments of peri-implant tissue destruction strongly suggests an important role for this mediator in the pathogenesis of peri-implant disease.

Our results show that IL-10 could be detected only in saliva of patients with peri-implant disease and it did not appear at detectable levels in saliva of healthy controls. It is known that IL-10 inhibit bone resorption by suppressing cyclooxygenase-2-dependent prostaglandin E2 synthesis (64, 65). IL-10 also inhibits recruitment of osteoclast precursors and their differentiation to mature multinucleated osteoclasts (66, 67). IL-10 response could be due to a specific Th1/Th2 response (68). In patients with periodontal disease, activation of immune response by *Porphyromonas gingivalis* produces reactive T cells, which respond by secreting IL-10 (69, 70). IL-10 is a cytokine inhibiting factor that regulates the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α and INF-γ (71, 72, 73, 23). Therefore, the elevated levels of IL-10 in patients with peri-implant disease could perform a relevant role in the regulation of local immune response (74, 75). Degree of its detectable level refers to more potent integrated immune response to try to maintain inflammatory balance. For example, as IL-10 is able to suppress excessive oxidative burst (Report of the American Academy of Periodontology 2002 (76)), its concrete levels in saliva reflect also a counteraction of tissues to profound oxidant stress events, known as significant actors in peri-implant disease (77).

Scientific evidence shows that expression of IL-10 and some other cytokines is time-dependent. Schierano et al. (78) assessed cytokines expression at different time-points after implant insertion. The results indicated that IL-10 expression increased at 4 month, decreased at 8 month and became almost undetectable at 12 month, suggesting that this cytokine combines to regulate immuno-inflammatory balance in peri-implant mucosa. The time factor was minimized in our study since all the patients had their endosseous implants in function for the period of 61 to 72 months (average time in function 67.2 ± 3.9 months).

Our study data indicated that elevated levels of IL-6 and IL-10 were statistically associated with positive BOP, GI ≥1 and PPD > 3 mm. Our investigation indicated that IL-6 and IL-10 levels are elevated in saliva of patients with peri-implant disease, as compared to healthy patients.

The data suggests that IL-6 levels > 1.5 pg/mg protein and and IL-10 levels > 1.0 pg/mg protein may be used as the basis of a diagnostic test for peri-implant disease in patients with implant-supported overdentures. Advantages of using saliva for such diagnostic purposes include ease of sample collection, the large volume of fluid available for study and simple assay procedure.

Although the results of present study demonstrate increased production of IL-6 and IL-10 in patients with peri-implant disease, the relationship of these findings with disease progression remains to be established in the futrure.

ACKNOWLEDGEMENTS

This investigation was supported in part by grant 5327 from the Estonian Science Foundation.

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CHARACTERIZATION OF THE ANTIOXIDANT PROFILE OF HUMAN SALIVA IN PERI-IMPLANT HEALTH AND DISEASE

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ABSTRACT

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Characterization of the antioxidant profile of human saliva in peri-implant health and disease.
Clin Oral Impl Res

Objectives
Peri-implant disease is considered to be an inflammatory disease, but many aspects of its pathogenesis remain unknown. At present, peri-implant disease is considered to be initiated and perpetuated by a small group of predominantly Gram-negative, anaerobic, or micro-aerophilic bacteria that colonize the sub-gingival area. Bacteria cause the observed tissue destruction directly by toxic products and indirectly by activating host defense systems, i.e. inflammation (Page & Kornman, 1997). A variety of molecular species appears in the

inflamed tissues, among them reactive species such as free radicals and reactive oxygen species (ROS).

The purpose of this study was to access levels of various antioxidants in saliva and identify differences between the saliva of patients with healthy peri-implant tissues and patients with peri-implant disease, examining whether the whole saliva of those with peri-implant disease conditions might have lower levels of antioxidants than that of healthy individuals.

Materials and Methods

30 healthy adult volunteers (14 men and 16 women) with implant-supported overdentures (Ankylos® Biofunctional implants, DentsplyFriadent, Germany) were selected from the group of patients from Tallinn Dental Clinic. Biochemical and clinical parameters evaluated were the following ones: the levels of urate, ascorbate, myeloperoxidase in saliva, total antioxidant status of saliva, pocket probing depth (mm), Gingival Index (0,1,2 or 3), and bleeding on probing (0 or 1).

Results and conclusion

Total antioxidant status (TAS) of saliva and concentration of uric acid and ascorbate, which are the main salivary antioxidants, are significantly decreased in patients with peri-implant disease. TAS in healthy subjects was 0.41 ± 0.10 for resting saliva and 0.31 ± 0.09 for stimulated saliva; in diseased subjects TAS was 0.19 ± 0.07 and 0.12 ± 0.03, respectively. In healthy subjects the concentration of urate was 307.2 ± 78.06 μM/l in resting saliva and 241.5 ± 89.09 μM/l in stimulated saliva. In diseased patients the concentration of urate was 120.0 ± 36.13 μM/l and 91.60 ± 39.35 μM/l, respectively. The concentration of ascorbate did not differ in resting and stimulated saliva. In healthy subjects it was 2.79 ± 0.81 mg/l and in diseased subjects it was 1.54 ± 0.30 mg/l. This may indicate that excessive ROS production in peri-implant disease is leading to the situation of excessive oxidative stress, which may be an important factor contributing the destruction of peri-implant tissues.

The importance of these findings may be the better understanding of the processes involved in the pathogenesis of peri-implant disease and that the treatment of peri-implant disease may involve adjuvant anti-oxidants supplementation together with cumulative interceptive supportive therapy concept introduced by Mombelli & Lang (1998).

KEY WORDS

Urate, ascorbate, total antioxidant status, oxidative stress, dental implants, diagnosis, saliva, peri-implantitis, peri-implant disease.

INTRODUCTION

Saliva is the first biological medium confronted by external materials that are taken into our body as part of food, drink, or inhaled volatile ingredients. During evolution, various defense mechanisms have developed in the saliva aimed at combating penetrating bacteria, viruses, or fungi and protecting against chemical or mechanical attack. Moreover, even after swallowing, saliva has mucosal protective capacity within the gastrointestinal tract (Rao et al. 1997; Marcozzi 1996). An extensive amount of research has been devoted to the immunological defense mechanism of saliva, primarily based on secretory IgA and the protein-enzymatic defense system, which, in turn, is based on the enzyme lysozyme and other components, such as histatin, lactoferrin, proline-rich protein, mucin, etc. The soft tissue integrity defense system, in which epidermal growth factor plays a pivotal role, has also been evaluated quite thoroughly (Rao et al. 1997).

Recently, the importance of another salivary defense system has become obvious. Similar to other biological systems, the salivary antioxidant system includes various molecules and enzymes, of which the most important are the uric acid molecule and the peroxidase enzyme, both of which are water-soluble. The lipid-soluble antioxidants carried by lipoproteins, whose concentration in saliva is very low, contribute no more than 10% of the total salivary antioxidant capacity (Meucci et al. 1998; Moore et al. 1994; Hirayama et al. 1997). Uric acid, the most important antioxidant molecule in saliva (Moore et al. 1994; Kondakova et al. 1999; Terao et al. 1993), contributes approximately 70% of the total salivary antioxidant capacity, according to Moore et al. (1994), with the antioxidant role of ascorbic acid molecule being secondary (Moore et al. 1994; Terao et al. 1993). Correlation between concentrations of uric acid in both saliva and plasma points the latter as the origin of salivary uric acid (Kondakova et al. 1999).

In the enzymatic salivary antioxidant system, peroxidase is by far the most important enzyme. Two peroxidase enzymes are found in saliva: salivary peroxidase, which resembles in structure and antigenic characteristics the lactoperoxidase found in bovine milk (Marcozzi 1996; Mansson-Rahemtulla et al. 1988; Mansson-Rahemtulla et al. 1990), and myeloperoxidase, produced by leucocytes in inflammatory regions of the oral cavity (Pruitt et al. 1990; Morrison et al. 1965; Revis 1977; Kowolik & Grant 1983; Smith & Yang 1984).

The involvement of reactive oxygen species (ROS) in periodontal pathology has been studied in last decades. Several studies (Asman et al. 1984, Henry et al. 1984, Shapira et al. 1991) have demonstrated that early onset forms of periodontitis are associated with functionally activated PMN exhibiting increased ROS production.

The detection of ROS oxidation products, the elevation of iron and copper ions, which catalyse the production of the most reactive free radical species, and the identification of an imbalance in the oxidant/antioxidant activity within

periodontal pockets, suggests a significant role for ROS in periodontal tissue destruction. In vitro studies have shown that ROS are capable of degrading a number of extracellular matrix components including proteoglycans, resulting in the modification of amino acid functional groups, leading to fragmentation of the core protein, whilst the constituent glycosaminoglycan chains undergo limited depolymerisation (Waddington et al. 2000).

Although extensive research has been done concerning the role of ROS and protective capacity of salivary antioxidants in periodontal disease, few have studied the role of excessive (high grade) oxidative stress in peri-implant disease. By our knowledge there are no studies regarding the role of excessive oxidative stress and salivary antioxidant system in the pathogenesis of peri-implant disease at present time.

Peri-implant disease is considered to be an inflammatory disease, but many aspects of its pathogenesis remain unknown. At present, peri-implant disease is considered to be initiated and perpetuated by a small group of predominantly Gram-negative, anaerobic, or micro-aerophilic bacteria that colonize the sub-gingival area. Bacteria cause the observed tissue destruction directly by toxic products and indirectly by activating host defense systems, i.e. inflammation (Page & Kornman, 1997). A variety of molecular species appears in the inflamed tissues, among them free radicals and reactive oxygen species.

Since all the patients participating in the study had no natural teeth (upper full denture and lower implant-supported overdenture wearers), we choose whole saliva as a medium for diagnostics. The overall advantages of using saliva as a diagnostic fluid have been recently reviewed (Streckfus & Bigler, 2002). The advantages of saliva compared to peri-implant sulcus fluid are (a) simple collection procedure and (b) non-invasive method of collection. Both issues were especially important concerning the age of patients (varied between 62 and 70 years). Saliva has been widely used for the quantification of different antioxidants and enzymes in oral cavity (Brock et al. 2004; Reznick et al. 2003; Nagler et al. 2000).

The purpose of this study was to access levels of various antioxidants in saliva and identify differences between the saliva of patients with healthy peri-implant tissues and patients with peri-implant disease, examining whether the whole saliva of those with peri-implant disease conditions might have lower levels of antioxidants than that of healthy individuals.

METHODS AND MATERIALS

Patient population

30 totally edentulous patients (62–70 years of age; 14 men, 16 women) with implant supported overdentures (Ankylos® dental implants, DentsplyFriadent, Germany), from the group of patients who were receiving maintenance care,

were selected to participate in this study. All the patients had two implants in lower jaw.

Prior to the start of the study the subjects gave their informed consent. Inclusion criteria were: (1) presence of 2 endosseous dental implants, (2) appropriate overdentures, (3) the same soft tissue biotype among all the patients, (4) healthy oral mucosa (no denture stomatitis etc), (5) no history of antibiotic treatment prior to the study for 3 months, (6) no history of medical conditions that required antibiotic prophylaxis, (7) negative history of chronic corticosteroid use, (8) no history of medication interfering with saliva secretion and (9) no history of taking nutritional supplements for 3 months. Since smoking adversely affects antioxidant capacity in biological fluids (Reznick et al. 2003; Nagler et al. 2000; Zappacosta et al. 1999), all patients included to the study were non-smokers.

A complete oral examination was given prior to sialometry and measurement of clinical parameters.

Clinical examination

The clinical evaluations were performed by single examiner (S.L.). The clinical examinations included assessment of peri-implant pocket probing depth (PPD) (mm), Gingival Index (GI) (0, 1, 2 or 3) (Löe et al. 1963) and bleeding on probing (BOP) (0 or 1). The pocket probing depth was measured to the nearest mm with a pressure-calibrated periodontal probe with a tip diameter of 0.5 mm and a probing force of 0.25 N (ClickProbe®, Hawe-Neos Dental, Bioggio, CH) (Lang et al. 1994, Karayannis et al. 1992).

Criteria for health and disease

Studied patients were categorized on the basis of pocket probing depth (PPD), gingival index (GI) and bleeding on probing (BOP) into two different groups as follows: patients with peri-implant disease (peri-implantitis and peri-implant mucositis) and healthy patients. The criteria have been referred to in previous reports (Pontoriero et al. 1994; Mombelli & Lang 1998). Briefly, peri-implantitis is defined as inflammation process around implants with loss of supporting bone and peri-implant mucositis is defined as reversible inflammation of the soft tissues with no loss of supporting bone.

The selection criteria were as follows: (1) in the healthy group both implants had no signs of peri-implant disease and in the peri-implant disease group both implants were affected with peri-implant disease (2) patients in healthy and diseased groups had to conform to the clinical parameters shown in Table 1.

Table 1. Clinical assessment and diagnosis of implants

Diagnosis	*PPD*	*GI*	*BOP*
Patients with peri-implant disease	≥ 4 mm	≥ 1	1
Healthy patients	≤ 3 mm	0	0

BOP, bleeding on probing; GI, gingival index; PPD, pocket probing depth

Sialometry

Unstimulated (resting) saliva specimens were obtained in the morning, and no oral stimulus was permitted for 90 min prior to collection. Saliva was collected before clinical evaluations were made. Whole saliva was collected by open suction method. Briefly, saliva was aspirated from the floor of the mouth into a test tube by a saliva ejector connected to the aspiration system. The ejector was gently swept around the subject's mouth in a circular motion with one pass in the buccal vestibules and around and under the tongue at approximately 15-s intervals for 5 min. Subsequently, a 2% citric acid solution was applied to the tongue dorsum bilaterally at 30-s intervals. After 2 min, glands were again stimulated while whole saliva was collected by the same method. Salivary gland flow rates are expressed as volume of saliva (ml) secreted per minute. The mean volume of saliva secreted (ml/min) under resting conditions was 0.43 ± 0.17 ml/min. Under stimulated conditions this value was 1.00 ± 0.16 ml/min.

All samples were assayed for blood contamination using an enzyme immunoassay kit for transferrin (Salimetrics). Transferrin is a large protein prevalent in blood at very high concentrations. Its large size prevents transferrin from being passively or actively transported from the general circulation into saliva. Saliva samples were assayed for transferrin using an enzyme imonoassay as described by Kivlighan et al. 2004. Contaminated samples were not included in the study.

Sialochemical analysis

Myeloperoxidase activity in saliva. MPO levels were measured with Bioxytech® MPO Enzyme immonoassay (Oxis International, Portland, USA). The MPO-EIA assay system is a "sandwich" ELISA. Antigen captured by a solid phase monoclonal antibody is detected with a biotin-labeled goat polyclonal anti-MPO. An avidin alkaline phosphatase conjugate then binds to the biotinylated antibody. The alkaline phosphatase substrate p-nitrophenyl phosphate (pNPP) is added and the yellow product (p-nitrophenol) is monitored at 405 nm.

Salivary uric acid concentration. Uric acid concentration was measured with a kit supplied by Roche Diagnostics GmbH (Mannheim, Germany). In this assay, uric acid was transformed by uricase into allantoin and hydrogen peroxide, which under the catalytic influence of peroxidase oxidized the cromogen (4-aminophenazone/N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline) to

form a red compound whose intensity of color was proportionalto the amount of uric acid present in the sample.

Salivary ascorbic acid concentration. Ascorbic acid concentration was measured according Ihara et al. (2000). Briefly, ascorbic acid is oxidized by 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy, free radical (TEMPO) to dehydroascorbic acid. The latter condenses with o-phenylenediamine (OPDA) to form a quinoxaline derivative that absorbs light at 340 nm. The change in absorbance at 340 nm is proportional to the concentration of ascorbic acid in specimen.

Salivary total antioxidative status (TAS). The assay used was based on a commercial kit supplied by Randox Laboratories (Ardmore, UK) in which metmyoglobin in the presence of iron was turned into ferrymyoglobin. Incubation of the latter with the Randox reagent 2,2´-azino-bis-[3-ethylbenzothiazoline sulfonate] (ABTS) resulted in the formation of a blue-green colored radical that could be detected at 600nm. Inhibition of the radical formation of $ABTS^{\bullet-}$ by antioxidant was calculated in millimolar quantities of ABTS disappearance.

Statistical analysis

For all variables mean and standard deviation (SD) for patients in different categories are presented. For statistical comparison between groups, Student's t-test was used. To archive approximate normal distribution, log-transformed values of myeloperoxidase levels were used for testing. All tests were conducted at conventional 5 % significance level. For statistical analysis software package R 2.0.0 for Windows (http://www.r-project.org) was used.

RESULTS

Patient data and clinical results

12 patients showed signs of peri-implant disease. A second group of 18 patients with healthy peri-implant tissues was used as control.

Table 2 shows patient demographics and clinical status of each group including PPD, gingival inflammation (GI) and BOP. Statistically significant differences for two groups were observed for all clinical parameters.

Table 2. Patient demographics (n=30) and clinical data

	Healthy (n=18)	***Diseased (n=12)***
Sex	8♂ and 10♀	6 ♂ and 6♀
Mean age (range)	66.0 ± 2.5 (62–70)	65.8 ± 2.2 (63–69)
PPD (mean ±SD)	2.63 (0.52)	3.85 (0.38)
% sites with		
gingival inflammation (GI ≥1)	0	100
bleeding on probing (BOP = 1)	0	100

BOP, bleeding on probing; PPD, probing pocket depth

Biochemical analysis of saliva

The total salivary antioxidant status is shown in *Table 3*, revealing a significantly lower activity in patients with peri-implant disease as compared with healthy subjects. Significant differences were observed both in resting and stimulated saliva ($p=0.0004$ and $p<0.0001$, respectively). The results of measurements of individual antioxidants in healthy subjects are shown in *Table 4*. Calculation of antioxidant production rates (per minute) showed that when salivary flow is taken into account, significantly more urate ($p<0.0001$), ascorbic acid ($p<0.0001$) and myeloperoxidase ($p<0.0001$) is secreted during salivary stimulation. The antioxidant composition of the saliva from patients with peri-implant disease was measured and summarized in *Table 5*. We also observed that under stimulation, significantly more urate ($p<0.0001$), ascorbic acid ($p<0.0001$) and myeloperoxidase ($p<0.0001$) are secreted in saliva, if salivary flow rate is taken into account.

Table 3. Total Antioxidant Status (TAS) of resting (R) and stimulated (S) saliva samples from healthy and diseased subjects

	TAS (mM/l) ± s.d.	
	R	**S**
Healthy	0,41 ± 0,10	0,31 ± 0,09
Diseased	0,19 ± 0,07	0,12 ± 0,03
p	0.0004	<0.0001

Table 4. The myeloperoxidase, uric acid and ascorbate concentrations and production rates in resting (R) and stimulated (S) saliva samples from healthy subjects

Saliva type	Uric acid (μM/l) mean ± s.d.	Uric acid (μM/min) mean ± s.d.	MPO (ng/ml) mean ± s.d.	MPO (ng/min) mean ± s.d.	Ascorbate (mg/l) mean ± s.d.	*Ascorbate* (mg/min) mean ± s.d.
R	307,20 ± 78,06	0,13 ± 0,03	15,03 ± 3,47	0,006 ± 0,001	2,79 ± 0,81	0,001 ± 0,001
S	241,50 ±89,09	0,24 ± 0,09	11,54 ± 1,46	0,012 ± 0,001	2,79 ± 0,81	0,003 ± 0,001

Table 5. The myeloperoxidase, uric acid and ascorbate concentrations and production rates in resting (R) and stimulated (S) saliva samples from subjects with peri-implant disease

Saliva type	Uric acid (μM/l) mean ± s.d.	Uric acid (μM/min) mean ± s.d.	MPO (ng/ml) mean ± s.d.	MPO (ng/min) mean ± s.d.	Ascorbate (mg/l) mean ± s.d.	*Ascorbate* (mg/min) mean ± s.d.
R	120,00 ±36,15	0,05 ± 0,02	50,62 ± 18,23	0,022 ± 0,008	1,54 ± 0,30	0,001 ± 0,000
S	91,60 ± 39,35	0,09 ± 0,04	26,16 ± 6,00	0,026 ± 0,006	1,54 ± 0,30	0,002 ± 0,000

Table 6 summarizes antioxidant status of saliva in healthy patients and patients with peri-implant disease. We found that total antioxidant status of saliva in patients with peri-implant disease is significantly lower as compared to healthy controls. Concentrations of uric acid and ascorbate in saliva of healthy patients were significantly higher compared to patient with peri-implant disease. At the same time, myeloperoxidase activity in patients with peri-implant disease is significantly increased if compared to healthy controls (Table 7).

Table 6. Antioxidant activity of saliva from healthy subjects and subjects with peri-implant disease.

Antioxidants	**Saliva**					
	Resting			***Stimulated***		
	Healthy	***Diseased***	***p***	***Healthy***	***Diseased***	***p***
Urate, μM/l	307,2	120,0	<0.0001	241,5	91,60	0.0006
Ascorbate, mg/l	2,79	1,54	0.003	2,79	1,54	0.003
TAS, mM/l	0,41	0,19	0.0004	0,31	0,12	<0.0001

TAS, Total Antioxidant Status

Table 7. Myeloperoxidase activity (ng/ml) of saliva from healthy subjects and subjects with peri-implant disease.

Saliva	MPO activity in saliva		
	Healthy	**Diseased**	***p***
Resting	15,0	50,6	0.001
Stimulated	11,5	26,2	0.0008

Discussion

Several studies indicated that there are increases in nitric oxide synthesis (Matejka et al. 1998), superoxide anion levels and myeloperoxidase activity (Cao & Smith 1989) in the inflamed periodontium. Reactive oxygen species (ROS) play important roles in physiological and immunoinflammatory reactions (Battino et al. 1999; Waddington et al. 2000). In the human body, there is an antioxidant mechanism to maintain the balance of oxidation-reduction (Teng 2003). The breakdown of this balance (i.e. increased ROS) could lead to increased damage directly by ROS. Indeed, several diseases have been correlated to an imbalance of oxidation-reduction or excessive oxidative stress (Macnee et al. 1999; Halliwell 1993; Liu & Mori 1999; Zhang et al. 2000). There are two possible causes for the excessive oxidative stress: increased ROS with no concomitant or less increased levels of antioxidants, or substantially decreased levels of antioxidants with no marked change of ROS. Enhanced superoxide anion production with no change of the antioxidant activity in gingival fluid of patients with chronic adult periodontitis, presumably due to the bacterial stimuli, has been reported (Guarnieri et al. 1991; Shapira et al. 1994). More recently, Brock et al (Brock et al. 2002; Brock et al. 2002) reported that antioxidant defense is reduced, and non-surgical therapy with improvements in clinical parameters can increase the antioxidant defense in chronic periodontitis patients. Similar results were reported in the recent study by Tsai et al. (2005).

The study of Sculley and Langley-Evans (2003) indicated that subjects with the worst periodontal health status tended to have greater oxidative injury. This is wholly consistent with the hypothesis that there is enhanced ROS-mediated damage to tissues in the most advanced states of periodontal disease (Sculley & Langley-Evans 2002). It is unlikely that oxidative processes play a causal role in the aetiology of periodontitis, but they are likely to contribute to disease progression unless abated through antioxidant action. Recent study (Sculley & Langley-Evans 2003) has demonstrated that subjects with advanced periodontitis and evidence of oxidative injury had the lowest TAS rates. This is believed to be the consequence of antioxidant depletion due to the ongoing free radical activity and destruction of scavenging antioxidant species.

Recent evidence shows that urate is the major antioxidant component in saliva (Sculley & Langley-Evans 2003; Lenander-Lumikari et al. 1998). Urate, ascorbate and albumin contribute most of the antioxidant protection in whole

saliva, but there is evidence of a salivary peroxidase enzyme too (Lenander-Lumikari et al. 1998). It is not clear whether the urate content of whole saliva directly reflects plasma concentrations or concentrations present in gingival crevicular fluid.

In the present study, two groups of patients were identified by clinical observations: a first group comprising patients with healthy dental implants and a second group including fixtures with inflammatory lesions. The current study included a rigorous pre-sampling protocol. Since smoking is known as a source of ROS, "reducer" of antioxidants (Pryor & Stone 1993) and risk factor for periodontal disease (Haber et al. 1993), only non-smokers were included to the study. Patients having whether two healthy or two diseased implants were included to the healthy and diseased groups, respectively. Strict clinical criteria were used to identify healthy and diseased groups; patients were identified as diseased only when all clinical parameters were showing signs of disease (PPD $\geq$ 4 mm, GI $\geq$ 1 and BOP =1) and as healthy only when all clinical parameters allowed identifying peri-implant health (PPD $\leq$ 3 mm, GI =0 and BOP =0).

Our study data indicated that MPO activity in saliva is increased in patients with peri-implant disease. These findings are similar to those of a previous study in which peri-implant sulcus fluid around diseased implants had higher MPO activity compared to healthy sites (Liskmann et al. 2004).

Total antioxidant status of saliva and concentration of uric acid and ascorbate, which are the main salivary antioxidants, are significantly decreased in patients with peri-implant disease. This may indicate that excessive ROS production in peri-implant disease is leading to the situation of excessive oxidative stress, which may be an important factor contributing the destruction of peri-implant tissues.

Local decreases in antioxidant capacity in peri-implant sulcus fluid are likely to be of greater significance in the aetiology of peri-implant disease and associated damage to the implant supporting structures than the more systemic changes we have noted in whole saliva. The presence of antioxidants bathing the peri-implant sulcus may be of major importance in dampening down inflammatory processes initiated by bacterial infection. Obtaining and analyzing peri-implant sulcus fluid samples is, however, a complex process requiring a degree of specialism. The results of this study suggest that whole saliva may contain simply measured indicators of oxidative processes and may provide a tool for the development and monitoring of new treatment strategies. The importance of these findings may be also that the treatment of peri-implant disease may involve adjuvant anti-oxidants supplementation together with cumulative interceptive supportive therapy concept introduced by Mombelli & Lang (1998).

The results of this investigation should be interpreted with caution due to the cross-sectional design. This particular study is of explorative value. We suggest that longitudinal monitoring of the antioxidant profile of saliva may confirm present results.

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Teadustöö

Peamiseks uurimisvaldkonnaks on implantaati ja hammast ümbritsevate pehmete ja kõvade kudede põletikulised kahjustused ülemäärase oksüdatiivse stressi valguses. Avaldanud 5 teadusartiklit ja 2 konverentsiettekannet