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ASSOCIATION BETWEEN PERIODONTAL AND SYSTEMIC INFLAMMATION

A STUDY OF PRO- AND ANTI-INFLAMMATORY MEDIATORS

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ASSOCIATION BETWEEN PERIODONTAL AND SYSTEMIC INFLAMMATION

A study of pro- and anti-inflammatory mediators

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium F202 of the Department of Pharmacology and Toxicology (Aapistie 5 B), on 22 November 2012, at 12 noon

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Abstract

The principal aim of this study was to explore associations between systemic inflammatory status and periodontal inflammation and tissue destruction. The study population consisted of 61 patients with chronic periodontitis, 30 periodontally healthy control subjects, and 80 subjects with type 1 diabetes mellitus (T1DM). The T1DM subjects were periodontally treated and re-examined eight weeks after completion of the treatment. The periodontal measures included plaque, probing depth (PD), bleeding on probing (BOP) and attachment level (AL). The serum levels of interleukin (IL)-6, tumor necrosis factor (TNF)-α, and interleukin (IL)-10, as well as the gingival crevicular fluid (GCF) level of matrix metalloproteinase (MMP)-8 were analyzed using commercially available ELISA assays, and serum high density lipoprotein (HDL) level using direct enzymatic methods.

Serum IL-6 level associated significantly with the extent of inflamed periodontal pockets in T1DM subjects. Moreover, serum IL-6 modulated local periodontal inflammation in T1DM patients; periodontal healing turned out to be poorer in subjects with a high level of serum IL-6 than in those with a low level. Serum TNF-α/IL-10 ratio was three times higher in chronic periodontitis patients than in periodontally healthy control subjects. In T1DM subjects a significant inverse association between serum HDL level and the extent of inflamed periodontal pockets was found; subjects with a low serum HDL level presented 50% more inflamed periodontal sites than subjects with a high serum HDL level. A significant association between GCF MMP-8 level in shallow crevices and the extent of periodontal attachment loss in chronic periodontitis patients was observed.

In conclusion, we focused on analyzing associations between systemic inflammatory status and periodontal conditions. According to our results, periodontal inflammation/infection is associated with systemic inflammatory status using serum IL-6, TNF-α/IL-10 ratio and HDL as indicators. Also, a high level of MMP-8 in GCF in shallow crevices could be indicative of higher susceptibility to periodontal infection and tissue destruction.

Keywords: bleeding on probing, cytokines, high density lipoprotein (HDL), inflammation, MMP-8, periodontitis, serum

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Tiivistelmä

Tutkimuksen tavoitteena oli tarkastella hampaiden kiinnityskudosten tulehduksen (pardodontiitti) ja siihen liittyvän inflammaation yhteyttä systeemiseen tulehdustilaan. Tutkimusaineistoon kuului 61 yleistervettä potilasta, joilla oli kohtalaisesti tai pitkälle edennyt parodontiitti, 30 yleistervettä yksilöä, joiden hampaiden kiinnityskudokset olivat terveet/lähes terveet (kontrolliryhmä), sekä 80 tyypin 1 diabetes mellitus (T1DM) potilasta, joilla esiintyi vaihtelevasti parodontiittia. T1DM potilaiden parodontiitti hoidettiin, ja heidät tutkittiin uudelleen kahdeksan viikon kuluttua hoidon päättymisestä. Tutkittavilta tarkastettiin plakin määrä, ientaskujen syvyys, ienverenvuoto ja hampaiden kiinnityskudoksen menetys. Systeemistä tulehdustilaa mitattiin käyttäen seerumin interleukiini (IL)-6, tuumorinekroosifaktori (TNF)-α ja interleukiini (IL)-10 tasoja. Lisäksi määritettiin ientaskunesteen matriksimetalloproteinaasi (MMP)-8 taso. Kaikki edellä mainitut tulehduksen välittäjäainetasot määritettiin käyttäen ELISA-menetelmää.

T1DM potilaiden seerumin IL-6 pitoisuuden ja tulehtuneiden ientaskujen määrän välillä vallitsi positiivinen yhteys. Lisäksi havaittiin, että seerumin korkea IL-6 taso heikensi parodontiitin paranemista. T1DM potilailla havaittiin käänteinen yhteys seerumin HDL-pitoisuuden ja tulehtuneiden ientaskujen määrän välillä. Tutkittavilla, joilla seerumin HDL-taso oli matala (<1.35 mmol/l), oli 50 % enemmän tulehtuneita ientaskuja kuin niillä, joilla HDL-taso oli korkea (≥1.35). Parodontiitti-ryhmässä seerumin TNF-α/IL-10 suhde oli kolminkertainen verrattuna kontrolliryhmän vastaavaan ilmentäen voimakkaampaa matala-asteista tulehdusta parodontiittipotilailla. Matalista (<4 mm) ientaskuista kerätyn ientasunesteen MMP-8 pitoisuuden ja parodontiitin vaikeusasteen välillä vallitsi merkittävä postiivinen yhteys parodontiitti-potilailla.

Yhteenvetona, systeemisen tulehdustilan ja hampaiden kiinnityskudosten välillä vallitsee kaksisuuntainen yhteys; toisaalta parodontiitti lisää matala-asteista systeemistä tulehdustilaa ja toisaalta kohonnut systeeminen tulehdustila lisää alttiutta parodontiittiin ja siihen liittyvään inflammaatioon. Lisäksi ientaskunesteen korkea MMP-8 pitoisuus matalissa ientaskuissa voi merkitä lisääntynyttä alttiutta parodontiumin alueen tulehdukselle ja kudostuholle.

Asiasanat: HDL-kolesteroli, ienverenvuoto, matriksimetalloproteinaasi MMP-8, parodontiitti, seerumi, sytokiinit

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Oulu, November 2012 Anna Norppa

Abbreviations

AL	attachment level
BL	bone loss
BMI	body mass index
BOP	bleeding on probing
DM	diabetes mellitus
GCF	gingival crevicular fluid
HbA1c	hemoglobin A1c
HDL	high-density lipoprotein
IL	interleukin
LDL	low-density lipoprotein
LPS	lipopolysaccharide
MMP	matrix metalloproteinase
PD	probing depth
Th	T helper cell
TIMP	tissue inhibitor of metalloproteinases
TNF	tumor necrosis factor
usCRP	ultrasensitive C-reactive protein

List of original articles

This thesis is based on the following original publications, which are referred to in this thesis by Roman numerals:

I Passoja A, Knuuttila M, Hiltunen L, Karttunen R, Niemelä O, Raunio T, Vainio O, Hedberg P & Tervonen T (2011) Serum interleukin-6 may modulate periodontal inflammation in type 1 diabetic subjects. J Clin Periodontol 38: 687–693.

II Passoja A, Puijola I, Knuuttila M, Niemelä O, Karttunen R, Raunio T & Tervonen T (2010) Serum levels of interleukin-10 and tumor necrosis factor-α in chronic periodontitis. J Clin Periodontol 37: 881–887.

III Passoja A, Knuuttila M, Hiltunen L, Karttunen R, Niemelä O, Vainio O, Hedberg P & Tervonen T (2011) Serum high-density lipoprotein cholesterol level associated with the extent of periodontal inflammation in type 1 diabetic subjects. J Clin Periodontol 38: 1071–1077.

IV Passoja A, Ylipalosaari M, Tervonen T, Raunio T & Knuuttila M (2008) Matrix metalloproteinase-8 concentration in shallow crevices associated with the extent of periodontal disease. J Clin Periodontol 35: 1027–1031.

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1 Introduction

Periodontitis is one of the most common oral health problems affecting adults worldwide (Dye 2012). While over 40–70% of dentate adults present poor periodontal health, measured as deepened periodontal pockets ≥ 4 mm, advanced forms of periodontitis have been reported to affect approximately 10–20% of the adult population according to national surveys in the USA, England, and Finland, respectively (Albandar 2002, Sheiham & Netuveli 2002, Suominen-Taipale *et al.* 2008).

Bacteria are essential in the initiation of periodontitis, but the progression and severity of the disease are thought to be determined by host immune responses in addition to behavioral and genetic factors. Moreover, recent studies have shown associations between periodontitis and systemic conditions, such as diabetes mellitus (Taylor & Borgnakke 2008, Colombo *et al.* 2011), cardiovascular diseases (D'Aiuto *et al.* 2006, Monteiro *et al.* 2009, Buhlin *et al.* 2011), and obesity (Ylöstalo *et al.* 2008, Han *et al.* 2010, Saxlin *et al.* 2011). The association between periodontal and systemic health is obvious; to what extent this association is spurious or causal is, however, unclear. Also, the direction of possible causal pathways is yet to be investigated. It has been shown that bacteria and their antigens from the periodontal area leak into the circulation, resulting in an elevated systemic inflammatory status also known as low-grade systemic inflammation (Kinane *et al.* 2005, Forner *et al.* 2006). On the other hand, less is known of the effect of systemic inflammation on periodontal health status.

Considering the high prevalence of periodontitis, a need to study its influence on general health exists. In this study we focused on the association between periodontal inflammation and tissue destruction and systemic inflammatory status, using both anti- and pro-inflammatory mediators/markers as indicators.

2 Literature review

2.1 Diagnosis of periodontal infection

Periodontal diagnosis can be based on clinical and radiological measurements, assessment of causal periodontal pathogens, or measurement of inflammatory markers in GCF or saliva.

Bleeding on probing (BOP) is a clinical parameter of an active, local inflammation. Probing depth (PD), the distance from the gingival margin to the base of the crevice/pocket, and attachment level (AL), the distance from the cementoenamel junction to the base of the crevice/pocket, are associated with connective tissue breakdown, which can further lead to resorption of the alveolar bone (bone loss, BL). In order to clinically study the progression of periodontal destruction, documentation of additional attachment or bone loss occurring between at least two time points is required. Healthy periodontal tissues and inflammation-derived tissue destruction are presented in Figure 1.

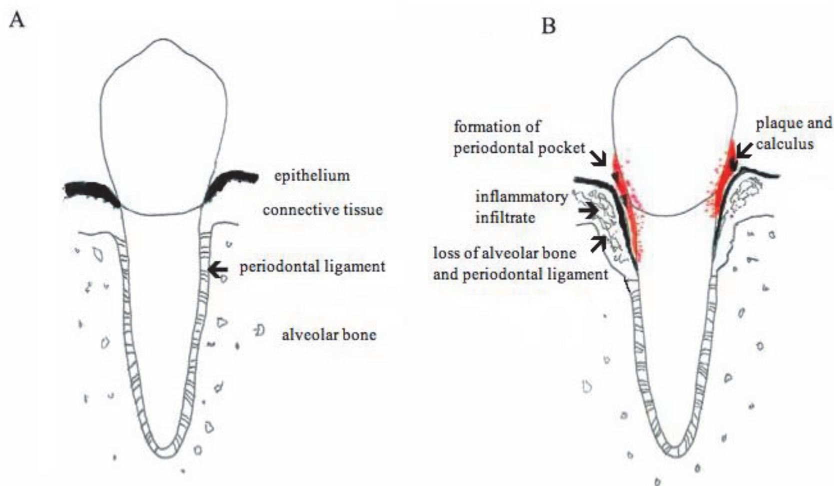

Fig. 1. A. Healthy periodontal tissues. 1B. Periodontal infection, inflammation-derived tissue destruction, and formation of a periodontal pocket.

Colonization of microbes and further, formation of subgingival biofilm, are prerequisites to the initiation and progression of chronic periodontitis. The initial colonization of microbes is comprised of mainly gram-positive cocci and rods, followed by an overgrowth of specific gram-negative species. Later on come fusobacteria and filaments, and finally, spirilla and spirochetes (Socransky & Haffajee 2005). Identification of bacterial species can be based on bacterial cultivation or, nowadays more often, genome-based methods. Despite the causative role of pathogens in the onset of the disease, its progression and severity are mainly determined by both innate and adaptive immune responses of the host. It appears that the inflammation-derived tissue destruction in periodontitis is induced by an overreaction of the immune response to periodontal pathogens.

During the development and progression of periodontal infection, a number of enzymes, tissue breakdown products, and inflammatory mediators (cytokines) are released from inflammatory and host tissue cells. Information regarding the activity of the disease can be drawn by analyzing biomarkers specific for inflammation, collagen degradation, and bone turnover. Gingival crevicular fluid (GCF) is widely used in studies concerning inflammatory biomarkers in relation to clinical periodontal status (Engebretson *et al.* 2002, Ribeiro *et al.* 2011), and efforts have been made to develop specific chairside tests to assess GCF biomarkers (Mäntylä *et al.* 2003). On the other hand, saliva contains GCF from all periodontal sites, providing an assessment of the overall health status of periodontal tissues (Wozniak *et al.* 2002) and has, therefore, been used as a diagnostic fluid when studying the biomarkers and host response mediators released during disease initiation and progression (Miller *et al.* 2006). The variability of host responses to periodontal pathogens can partly be explained by differences in the production of bioactive molecules as well as by environmental and genetic factors (Gemmel *et al.* 2007, Kinane *et al.* 2007).

2.2 Low-grade systemic inflammation – a link between periodontal infection and systemic diseases

According to a number of studies, associations exist between periodontal infection and systemic health conditions, the association between diabetes mellitus and periodontitis being the best documented (Lalla *et al.* 2007, Taylor & Borgnakke 2008, Colombo *et al.* 2011). Also, the association between periodontitis and cardiovascular diseases has been shown (D'Aiuto *et al.* 2006, Monteiro *et al.* 2009, Buhlin *et al.* 2011). That chronic periodontitis, despite its

local nature, is related to systemic conditions has previously been confirmed by the finding of an association between periodontitis and obesity (Ylöstalo *et al.* 2008, Khader *et al.* 2009, Saxlin *et al.* 2011). So far, the exact mechanisms behind these associations are unclear.

Inflammation is a complex defense mechanism in which immune cells together with other host cells are activated to produce inflammatory mediators that guide an amplifying cascade of biochemical and cellular events. Inflammatory reactions are furthermore driven and maintained by a network of bioactive cytokines and chemokines (Gabay 2006, Kinane *et al.* 2007). Periodontal pathogens and/or their products are known to leak from the inflamed area into the circulation (Kinane *et al.* 2005, Forner *et al.* 2006) to challenge the immune system, i.e. the circulating and resident immune cells in the body. Confirming this, periodontal bacteria DNA (Haraszthy *et al.* 2000) and viable pathogens (Kozarov *et al.* 2005) have been detected in human atherosclerotic plaques.

The relationship between periodontal infection and systemic diseases may be causal, in which case the systemic low-grade inflammation generated by periodontal infection predisposes to systemic disease conditions (D'Aiuto *et al.* 2006, Pussinen *et al.* 2007). On the other hand, chronic periodontitis and systemic diseases, such as cardiovascular diseases and type II diabetes mellitus, share many common risk factors, including overweight (Ylöstalo *et al.* 2008, Khader *et al.* 2009, Zalesin *et al.* 2011) and smoking (Bergström 2006, Leone *et al.* 2010). Both smoking and overweight are known for their ability to increase individuals' systemic inflammatory burden (Ritchie 2007, Reichert *et al.* 2009), which may be the biological background for the spurious associations between periodontal infection and systemic diseases. Thirdly, the systemic inflammatory burden generated by a systemic condition may increase individuals' susceptibility to local periodontal inflammation.

2.3 Host responses in chronic periodontitis

In addition to known risk factors, such as smoking and overweight, genetic factors are known to play a role in susceptibility to periodontal disease. Genetically transmitted traits such as gene polymorphisms may regulate the host's inflammatory response to a bacterial challenge (Michalowicz *et al.* 2000, Burgner *et al.* 2006). The biological background behind the association between periodontal disease and cytokine gene polymorphisms is that carriage of certain

alleles in cytokine genes, like IL-1, TNF-α, and IL-6, is related to increased production of the cytokine (Loos *et al.* 2005, Shapira *et al.* 2005, Takashiba & Naruishi 2006). For instance, subjects carrying the GG genotype of *IL-6*-174 are known to present higher levels of serum IL-6 (Fishman *et al.* 1998, Raunio *et al.* 2007), and furthermore, the same genotype has been reported to be associated with the extent of periodontitis in otherwise healthy periodontitis patients (Nibali *et al.* 2008) and in subjects with type 1 diabetes mellitus (Raunio *et al.* 2009).

Periodontal health has been described as a dynamic state in which the pro- and anti-inflammatory responses of the host are in balance to prevent unwarranted inflammation and subsequent tissue destruction (Gaffen & Hajishengallis 2008). Under pathologic inflammatory conditions, such as periodontitis, the balance between pro- and anti-inflammation has been converted into pro-inflammatory activity. In this respect, periodontal inflammation seems to be similar to several systemic inflammatory conditions, such as myocardial infarction (Karpinski *et al.* 2009), alcoholic liver disease (McClain *et al.* 2004), inflammatory bowel disease, and colorectal cancer (Szkaradkiewicz *et al.* 2009), in which a predominance of pro-inflammatory activity is observed. Such a shift could be a result of environmental influences leading to an increased number of pathogenic bacteria, depression of the host's defense mechanism, or both (Gemmel *et al.* 2007). While pro-inflammatory responses include production of pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1, which are able to enhance both local and systemic inflammation (Gemmel *et al.* 1997), the anti-inflammatory cytokines IL-10 and IL-4, on the other hand, tend to attenuate the inflammatory response (Moore *et al.* 2001). These cytokines, among other inflammatory mediators, guide further immune responses including innate and adaptive responses. Based on earlier literature, the pro-inflammatory mediators IL-6, TNF-α, and MMP-8, and the anti-inflammatory mediators IL-10 and HDL, under study here, are known to play a central role in periodontal inflammation.

2.3.1 Innate immune responses

Innate immune responses are part of the inherent biological defense response during the initial hours and days of infection (Kinane *et al.* 2007). They are activated after the capacity of primary defenses, such as saliva, specialized epithelial tissues, and commensal microflora, are breached. Recruitment of immune cells, complement activation, identification and removal of infectious agents, and activation of the adaptive immune response are the main actions of

the innate immunity response (Van Dyke & Kornman 2008). During the innate response, immune cells become activated to produce cytokines by bacterial stimulus, the predominant cell type being PMN leukocytes (Ryan & Majno 1977). In addition, monocytes/macrophages, dendritic cells, mast cells, eosinophils, and so-called NK cells bear innate immunity responses. Furthermore, direct contact with bacteria or increased levels of pro-inflammatory cytokines promote the production of inflammatory mediators by fibroblasts, keratinocytes, and endothelial cells (Janeway & Medzhitov 2002, Taylor 2010). It should be noted that the local reactions of innate immunity can also have an influence on systemic inflammatory status.

Bacteria and their products, such as lipopolysacharide (LPS), trigger a host response, for instance, through host cell recognition of microbial-associated "patterns", e.g. bacterial lipopolysaccharide, DNA, and fimbriae (Krieg 2000, Beutler 2002, Taylor 2010). Host cell recognition of microbial structures relies on diverse families of receptors. These receptors, including Toll-like receptors, allow detection of infectious agents and provide signals that further activate defense mechanisms, such as opsonization, activation of complement and coagulate cascades, phagosytosis, activation of pro-inflammatory signaling pathways, and induction of apoptosis (Janeway & Medzhitov 2002). In addition, Toll-like receptors have an essential role in the initiation of adaptive immune responses (Beutler 2002, Janeway & Medzhitov 2002). Moreover, CD14, another host cell recognition receptor, is found on the cell membrane of monocytes/macrophages and neutrophils, and is involved in the recognition of microbial-associated patterns. It acts on the cell membrane by retaining LPS and other bacterial ligands and facilitating ligand-Toll-like receptor interaction, resulting in the release of cytokines from the cell (Wright *et al.* 1990, Medzhitov & Janeway 2000). Moreover, macrophages express several other pattern recognition receptors, such as macrophage mannose receptor (MMR) and macrophage scavenger receptor (MSR) (Janeway & Medzhitov 2002).

2.3.2 Adaptive immune responses

Adaptive immune responses are integrated with innate immunity through the action of cytokines, such as IL-1 and TNF-α, which amplify the inflammatory reaction and enhance leukocyte migration (Yamamoto *et al.* 2006, Taylor 2010). In addition, adaptive responses are activated by antigenic processing and presenting as well as proliferation and differentiation of effector cells (Hornef *et*

al. 2002). Dendritic cells are pivotal in the activation of adaptive responses. Toll-like-receptor-mediated pathogen recognition promotes dendritic cell maturation, which includes expression of co-stimulatory molecules, antigen processing, increased major histocompatibility complex (MHC) molecule expression, production of cytokines/chemokines, and migration to the lymph node. The activation of dendritic cells is critical for naïve antigen-specific T-cell priming and differentiation (Banchereau & Steinman 1998).

T cells have a fundamental role in periodontal diseases. They are functionally divided into two main classes: cytotoxic T cells that express CD8 molecule on their cell surface, and helper T (Th) cells that express CD4 molecule (Gemmell *et al.* 2007). CD4+ T lymphocytes are responsible for the qualitative profile of cytokines. Antigen stimulation promotes naïve CD4+ T cells to further proliferate and differentiate into different effector cells—Th1, Th2, and Th17 cells—characterized by the production of specific cytokines and effector functions (Mosmann & Coffman 1989). The Th1 cells secrete mainly IFN-γ (essential for activation of macrophages, NK-cells, and CD8+ T cells) and IL-2, but also IL-1β, IL-12, and TNF-α, which induce the cell-mediated immune response. These cytokines further stimulate effector T cells (i.e. CD8+ cells) and to some extent B cells. In contrast, humoral immunity is mediated by Th2 cells, which express IL-4, -5, -6, -10, and -13. In periodontitis, Th1/Th2 dominance is not very clear, and controversial results have been presented as to whether Th1 cells are associated with a stable periodontal lesion and Th2 response is pronounced in disease progression (Gemmell *et al.* 2001, Gaffen & Hajishengallis 2008). A new subset of CD4+ T cells are the Th17 cells that produce mainly IL-17, which is involved in an initial inflammatory response by promoting rapid recruitment of neutrophils (Harrington *et al.* 2005). Cytotoxic CD8+ T cells are principally associated with defense against viral infections, but they play a role in bacterial infections, as well. CD8+ T cells recognize infected cells that express pathogen-derived peptide complexes with MHC class I molecules on their surfaces and kill them. They also express various effector molecules that mediate defense against pathogens (Wong & Pamer 2003).

Regardless of the regulatory action of T cells, B cells serve as an important and well-controlled part of the adaptive immune response. B cells are activated by cytokines produced predominantly by Th2 cells (Yamamoto *et al.* 1997) or by direct contact with antigens (Gemmel *et al.* 2007). Activated B cells transform into plasma cells, which produce immunoglobulins that identify and bind to antigens (Berglundh *et al.* 2007). In addition, B cells contribute to the activation

of the immune system and exhibit important immunoregulatory functions, including direct and indirect effects on other cells through antigen presentation and production of cytokines (Porakishvili *et al.* 2001, Lund *et al.* 2005). Up-regulation of the adaptive immune response helps clear the infection and builds specific immunity with a memory component, therefore being more precise than the innate immune response (Graves & Cochran 2003).

2.4 Pro-inflammatory biomarkers

2.4.1 Interleukin (IL)-6

IL-6 is a multifunctional cytokine that possesses a wide range of activities. In inflammation, IL-6 plays a key role by regulating the immune response, hematopoiesis, and the acute phase response. It is produced by a variety of cell types, including monocytes/macrophages, T and B cells, keratinocytes, endothelial cells, adipocytes, fibroblasts, and several tumor cells. The most important sources in periodontal diseases are macrophages and monocytes at the site of inflammation (Kishimoto 2006, Gabay 2006, Palmqvist *et al.* 2008). Bacterial antigens or other cytokines (IL-1β, TNF-α) trigger local production of IL-6 (Ishihara & Hirano 2002, Palmqvist *et al.* 2008), also in inflamed periodontal tissue (Takahashi *et al.* 1994).

During innate immunity reactions IL-6 activates inflammatory cells, including macrophages, lymphocytes, and hepatocytes, to produce acute phase proteins like C reactive protein (CRP) and amyloid A, markers for cardiovascular diseases (Pearson *et al.* 2003, Gabay 2006). CRP and serum amyloid A are mainly produced by the liver and enhance the immune response further by activating complement cascade, inducing production of pro-inflammatory cytokines and stimulating neutrophile chemotaxis (Gabay 2006, Cronstein 2007). Furthermore, IL-6 plays a central role in the development of specific cellular and humoral immune responses, including end-stage B cell differentiation, immunoglobulin secretion, and T cell activation. IL-6 is important in the activation of the adaptive immune system; it amplifies monocyte accumulation at the site of inflammation through a shift in local chemokine production (Kaplanski *et al.* 2003). The biological activities of IL-6 also include the ability to increase bone resorption by increasing osteoclast formation both alone and together with other bone-resorbing agents (Ishimi *et al.* 1990, Tamura *et al.* 1993).

Elevated levels of serum IL-6 have been reported in many autoimmune diseases, such as rheumatoid arthritis and psoriasis (Kishimoto 1992, Ishihara & Hirano 2002). In addition, IL-6 is highly expressed in acute and chronic inflammatory states, like sepsis and atherosclerosis (Schluter *et al.* 2002, Beckman *et al.* 2005).

IL-6 is known as one of the key cytokines in chronic periodontitis (Nibali *et al.* 2012). Earlier studies have suggested that a significant association exists between periodontal destruction and a high level of IL-6 in GCF (Geivelis *et al.* 1993), and that patients with chronic periodontitis present higher serum IL-6 levels than healthy controls (Buhlin *et al.* 2003), or increased IL-6 levels are detected with increasing severity of periodontal disease (Raunio *et al.* 2007). Regarding periodontal infection, a high serum IL-6 level may have a specific role in modulating local inflammatory responses in individuals with diabetes mellitus (Andriankaja *et al.* 2009).

According to the review by Irwing & Myrillas (1998), IL-6 has been found to also exert anti-inflammatory properties, e.g. enhancement of tissue inhibitor of metalloproteinase (TIMP) production and suppression of pro-inflammatory cytokines IL-1β and TNF-α. In addition, down-regulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory molecules (e.g. IL-1 receptor antagonist, TNF soluble receptor) by IL-6 in acute inflammatory processes have been reported (Xing *et al.* 1998). However, the balance between the protective and destructive activities of IL-6 in periodontitis is yet to be determined (Irwin & Myrillas 1998).

2.4.2 Tumor necrosis factor (TNF)-α

TNF-α amplifies innate host responses and may be regarded as an "early" cytokine. By inducing up-regulation of adhesion molecules and stimulating the production of chemokines, TNF-α enhances recruitment of inflammatory cells to the site of inflammation (Dinarello 1996, Pfizenmaier *et al.* 1996). The initial production of TNF-α by monocytes/macrophages, PMN leukocytes, fibroblasts (of gingival and periodontal ligament origin), epithelial and endothelial cells, and odontoblasts occurs as a result of bacterial stimulation (Graves *et al.* 2001, Yamamoto *et al.* 2006), and along with IL-1β, it further enhances these cells to produce IL-6 (Palmqvist *et al.* 2008). In addition, adipose tissue (Ritchie 2007) and multiple immune cells in the liver (Kupffer cells, hepatocytes, and T cells) are known for their capacity to produce TNF-α (Tiegs & Loshe 2010).

Systemic activities of TNF-α, to mention a couple, include stimulation of hepatocytes to produce CRP (Yudkin *et al.* 2000) and playing a predominant role in the development of insulin resistance in obese subjects (Nishimura & Murayama 2001).

Regarding periodontal tissue destruction, TNF-α stimulates fibroblasts to produce matrix metalloproteinases (MMPs) and therefore amplifies destruction of the collagen matrix (Meikle *et al.* 1989). In addition, TNF-α enhances bone loss by stimulating osteoclastic bone resorption and inhibiting bone collagen synthesis (Hong *et al.* 2004, Lima *et al.* 2004). At that, TNF-α stimulates apoptosis of fibroblasts, resulting in limited repair of the periodontal tissues (Graves *et al.* 2001). On the other hand, the results of animal studies suggest that use of TNF-α antagonists results in a reduction of inflammatory cell recruitment and periodontal tissue destruction (Delima *et al.* 2001).

There is some evidence that a positive association exists between the level of circulating TNF-α and periodontal inflammation (Engebretson *et al.* 2007, Andrukhov *et al.* 2011). Further supporting the concept of an association between local infection (periodontitis) and systemic inflammatory burden, a reduction in serum TNF-α level after non-surgical periodontal treatment has been reported in diabetic patients (Correa *et al.* 2010, Dag *et al.* 2009).

2.4.3 Matrix metalloproteinase (MMP)-8

Although pathogenic microflora produce lytic enzymes, it is likely that bacteria-derived enzymes and cytotoxins are not solely responsible for tissue breakdown in periodontitis (Genco *et al.* 1999). Most periodontal destruction results from activated host proteases like matrix metalloproteinases (MMPs), which also participate in physiological development and remodeling of tissues (Sorsa *et al.* 2004, Uitto *et al.* 2003). In addition to periodontitis, increased levels of MMP-8 are associated with many diseases, including bronchiectasis, (Prikk *et al.* 2001), asthma (Prikk *et al.* 2002), atherosclerosis (Tuomainen *et al.* 2007), inflammatory bowel disease (Pirilä *et al.* 2003), oral cysts (Wahlgren *et al.* 2001), and oral cancer (Moilanen *et al.* 2002).

MMPs are all structurally related but genetically distinct proteases, and they differ in terms of substrate specificity. Collagenases (MMP-1, -8, -13) degrade mainly type I and III collagens; type IV collagenases/gelatinases (MMP-2, -9) act on basement membrane components and partially degraded collagen; stromelysins (MMP-3, -10, -11) and matrinolysins (MMP-7) have wide substrate

specificity; metalloelastase (MMP-12) degrades mainly elastin, and membrane-type MMPs (MMP-14, -15, -16, -17, -24, -25) are integral trans-membrane proteins (Morgan *et al.* 2011). While the main sources of MMP-8 are PMN leukocytes, it is also secreted by epithelial cells, plasma cells, and fibroblasts (Sorsa *et al.* 1992). MMP-8 is produced as a latent, non-active form and secreted into the extracellular matrix, and is activated by a so-called cystein switch (Sorsa *et al.* 2004). In addition to activation of the latent enzyme, production of MMPs is regulated in several ways, including synthesis of the proenzyme (gene expression) and the presence of inhibitory proteins (tissue inhibitor of metalloproteinases, TIMPs) (Birkedal-Hansen 1993, Raza & Cornelius 2000). An imbalance between TIMPs and MMPs results in unwarranted connective tissue destruction, as seen in periodontitis (Biyikoglu *et al.* 2009). A bacterial stimulus can activate PMN cells to release MMP-8, and pro-inflammatory cytokines (IL-1β and TNF-α) can stimulate non-PMN-lineage cells in the periodontal area to produce MMP-8 (Sorsa *et al.* 2004, Ozcaka *et al.* 2011).

The amount and activity of MMP-8 are highly increased in GCF in diseased periodontal pockets and correlated with the severity of periodontal disease (Kinane *et al.* 2003, Uitto *et al.* 2003, Marcaccini 2010). Besides inflammation, smoking has been shown to increase the expression of MMP-8 in periodontal tissue (Liu *et al.* 2006), although contrary data suggest that smokers present lower GCF MMP-8 levels than non-smokers (Mäntylä *et al.* 2006). Periodontal treatment (scaling and root planing) has been documented to decrease MMP-8 levels and activity (Kinane *et al.* 2003, Marcaccini *et al.* 2010), and poor response to treatment has been associated with persistently elevated MMP-8 levels (Mäntylä *et al.* 2006).

2.4.4 Other pro-inflammatory biomarkers

Pro-inflammatory cytokine IL-1β is a multifunctional cytokine that regulates various cellular and tissue functions. It is mainly expressed by macrophages and dendritic cells in periodontal inflammation, but gingival fibroblasts, periodontal ligament cells, and osteoblasts also produce it (Liu *et al.* 2010). IL-1β regulates inflammatory reactions in numerous ways, like increasing the production of IL-6, decreasing the production of IL-10 (Deschner *et al.* 2000), and enhancing the expression of matrix metalloproteinases from gingival fibroblasts and periodontal ligament cells (Cox *et al.* 2006). Furthermore, IL-1β is a potent stimulator of bone

resorption, participating in multiple steps of osteoclast recruitment, like differentiation, multinucleation, activation, and survival (Nakamura & Jimi 2006).

Of other pro-inflammatory mediators, prostanoids, particularly prostaglandin E_2, are important. Prostaglandin E_2 levels are increased in inflamed periodontal tissues and GCF in periodontitis patients (Offenbacher *et al.* 1986). In addition, the GCF level of prostaglandin E_2 is effective in predicting the progression of periodontal destruction (Offenbacher *et al.* 1986).

2.5 Anti-inflammatory biomarkers

2.5.1 Interleukin (IL)-10

IL-10 is known to affect several steps in antimicrobial immunity, and its main functions are to limit and ultimately terminate inflammatory responses and regulate the differentiation and proliferation of immune cells such as T cells, B cells, NK cells, and mast cells (Moore *et al.* 2001, Asadullah *et al.* 2003).

The immunoregulatory cytokine IL-10 suppresses pro-inflammatory cytokine production and the antigen-presenting capacity of monocytes/macrophages and dendritic cells, and therefore indirectly reduces cytokine production by T cells and natural killer (NK) cells (Fiorentino *et al.* 1989, de Waal Malefyt *et al.* 1991). Thus, IL-10 inhibits the production of pro-inflammatory cytokines by inhibiting MHC class II expression in monocytes and macrophages and directly inhibiting the proliferation of CD4+ T cells (Joss *et al.* 2000, Moore *et al.* 2001). IL-10 favors the phagocytic activity of monocytes and macrophages (Buchwald *et al.* 1999) and regulates innate and adaptive Th1 and Th2 responses by limiting T cell activation and differentiation in the lymph nodes (Moore et al. 2001). In addition, IL-10 prevents apoptosis of B cells and enhances their proliferation and differentiation towards plasma cells (Levy & Brouet 1994, Rousset *et al.* 1995).

The major sources of IL-10 are the macrophages, but activated T cells, dendritic cells, monocytes, B cells, and periodontal ligament cells also produce it (Deschner *et al.* 2000, Sabat *et al.* 2010). The production is stimulated by several endogenous and exogenous factors such as endotoxin, TNF-α, and catecholamines (Platzer *et al.* 2000, Riese *et al.* 2000). The production of IL-10 in gingival tissue is enhanced during acute inflammation (Kobayashi *et al.* 2011).

By inhibiting immune responses, IL-10 may diminish clearance of infectious pathogens (Couper *et al*. 2008). Resistance to infection can nearly always be

improved by reducing IL-10 levels. Furthermore, even normal levels of IL-10 tend to limit the effectiveness of antipathogenic immune responses, especially innate immunity and adaptive Th1 responses (Moore *et al.* 2001). Higher levels of serum IL-10 have been reported with infectious diseases, such as malaria (Peyron *et al.* 1994), filiariasis (Mahanty 1997), tuberculosis (Gerosa *et al.* 1999), and candidiasis (Roilides *et al.* 1998). On the contrary, a deficiency of IL-10 is of pathophysiological relevance in chronic inflammatory disorders (Asadullah *et al.* 2003). Regarding atherosclerosis, elevated serum IL-10 levels are predictive of a better clinical outcome after acute coronary syndrome, and neutralize the increased risk associated with elevated serum levels of CRP (Heeschen *et al.* 2003).

Resolution of infection requires a coordinated response in which initial pro-inflammatory mechanisms clear the pathogen and are then down-regulated by IL-10 before uncontrolled pathology occurs. Excessive activation of the immune system may result in severe complications. In chronic infection, such as periodontitis, suppression of the immune system prevents tissue damage (Couper *et al.* 2008), and a lack of IL-10 has been linked to accelerated alveolar bone resorption (Al-Rasheed *et al.* 2003).

2.5.2 High density lipoprotein (HDL)

High-density lipoproteins (HDLs) are the smallest (7.0–12 nm in diameter) and densest (1.063–1.210 g/ml) of the plasma lipid-protein complexes. HDL particles are composed of an outer layer comprised of phospholipid, free cholesterol, and various apolipoproteins (Apo), which cover the hydrophobic core containing triglycerides and cholesterol esters (Barter *et al.* 2003). The main HDL apolipoprotein is Apo A-I, accounting for about 60% of the protein content of HDL. It is synthesized in the intestines and liver, and considered largely responsible for the antiatherogenic effects of HDL (Shah *et al.* 2001). HDL particles also provide a transport vehicle for other plasma proteins, such as cholesteryl ester transfer protein, cholesterol acyltransferase, and phospholipid transfer protein (Barter 2003).

HDL is known to down-regulate inflammation via several biological pathways. Accordingly, HDL has been found to limit cytokine expression by binding and neutralizing lipopolysacharide (LPS) of Gram-negative bacteria (Levine *et al.* 1993, Nofer *et al.* 2002). Furthermore, HDL is known to inhibit LDL-induced monocyte chemotactic activity in arterial wall co-cultures (Navab *et*

al. 1991). The antiatherogenic function of HDL is linked to its ability to promote the efflux of excess cholesterol by macrophages and non-macrophages and return it to the liver for excretion (reverse cholesterol transport) (Barter & Rye 1996). HDL is also considered to have an antioxidant feature; treatment of human endothelial cells with HDL or apoA-I (main constituent of HDL cholesterol) converted the cells unable to oxidize LDL (Navab *et al.* 2000). The protective role of HDL may be related not only to the level, but also to the quality of HDL (Khera *et al.* 2011). Supporting the previous, Pussinen and co-workers (Pussinen *et al.* 2004) showed in an in vitro study that HDL-mediated cholesterol efflux tended to be higher after periodontal treatment, suggesting an improved protective capacity of HDL after elimination of infection.

Aside from being known for its anti-inflammatory and anti-infective activities in protecting against cardiovascular diseases (Barter *et al.* 2003), low levels of serum HDL have been associated with other systemic inflammatory conditions such as vascular dementia (Zuliani *et al.* 2001) and Alzheimer's disease (Reiss *et al.* 2004).

That an association exists between periodontal infection and serum lipid profile was supported by longitudinal studies where decreased levels of serum low-density lipoprotein (LDL) and total cholesterol were found after intensive periodontal therapy in severely affected periodontitis patients (D'Aiuto *et al.* 2005, D'Aiuto *et al.* 2006). Compared with periodontally healthy subjects, chronic periodontitis patients have been reported to have a lower serum HDL level and ratio of HDL to total cholesterol (Buhlin *et al.* 2003, Nibali *et al.* 2007, Monteiro *et al.* 2009). On the other hand, a significant increase in the level of serum HDL along with successful periodontal intervention has been reported (Pussinen *et al.* 2004, Buhlin *et al.* 2009).

2.6 Diabetes mellitus

Diabetic subjects are known to have an exaggerated inflammatory response and an increased systemic inflammatory burden, making them an interesting target population for studying associations between local and systemic inflammation. The term "diabetes mellitus" describes a heterogeneous group of disorders characterized by elevated levels of blood glucose and abnormalities in carbohydrate, lipid, and protein metabolism (Nassar *et al.* 2007). Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease in which the insulin-producing β-cells of the pancreas are destroyed by the immune system, resulting in

hyperglycemia due to the lack of insulin. Type 2 diabetes mellitus (T2DM) is caused by resistance to insulin combined with a failure to produce enough additional insulin to compensate for the insulin resistance, and it is commonly linked with obesity, which promotes insulin resistance (Kahn & Flier 2000). The association between diabetes mellitus and periodontitis is widely studied and well documented. A number of periodontal-inflammation-associated pathological mechanisms related to elevated blood glucose levels have been defined, including activation of the sorbitol pathway and formation of advanced glycosylation end products (AGEs) (Lamster *et al.* 2008). Hyperglycemia is also known to induce production of reactive oxidative species (Brownlee 2001), resulting in damage to many biological molecules (DNA, lipids, protein) and leading to tissue destruction (Nassar *et al.* 2007). There is convincing evidence that free oxygen radicals and oxidative stress play a role in the development of diabetic organ complications (Matough *et al.* 2012). Systemic level oxidative stress has been associated with severe periodontitis in non-diabetics (D'Aiuto *et al.* 2010) and according to a recent study, T2DM subjects with periodontitis present lower systemic antioxidant capacity and higher oxidative stress than T2DM subjects without periodontitis, indicating a negative effect of periodontitis on the already compromised systemic oxidative status of T2DM subjects (Allen *et al.* 2011).

Hyperglycemia as such is known to induce a systemic pro-inflammatory state (Devaraj *et al.* 2007, Kaul *et al.* 2010). The major causative factor behind the micro-vascular (retinopathy, nephropathy, and neuropathy), and macro-vascular (cardiovascular, cerebrovascular, and peripheral vascular diseases) complications of diabetes mellitus is long-term hyperglycemia (Kaul *et al.* 2010), but growing evidence suggests that diabetic complications are also linked with systemic inflammation (Devaraj *et al.* 2007, Saraheimo *et al.* 2003). Both gingivitis and periodontitis are also considered hyperglycemia-related complications (Taylor 2001, Taylor & Borgnakke 2008) and they can appear already in childhood and adolescence (Lalla *et al.* 2007). T1DM patients have been reported to present increased gingival inflammation to a comparable bacterial challenge when compared with non-diabetic subjects (Salvi *et al.* 2005). The background for the previous is an exaggerated immune response resulting in increased production of pro-inflammatory mediators in the gingival area in T1DM subjects (Salvi *et al.* 2010). Also, in an earlier *in vitro* study, monocytic release of IL-6, IL-1β, and TNF-α was significantly increased in diabetic patients compared with healthy subjects (Salvi *et al.* 1997, Devaraj *et al.* 2007).

Regarding serum lipid profile, T1DM is known to alter lipid metabolism and quantitative lipid abnormalities are observed in T1DM patients with poor glycemic control (increased plasma triglyceride and LDL levels) or nephropathy (increased plasma triglycerides and LDL, decreased HDL levels). In addition, several qualitative abnormalities, such as increased cholesterol/triglyceride ratios in very-low-density lipoprotein (VLDL), increased triglycerides in LDL and HDL, glycation of apolipoproteins, and increased oxidation of LDL, are observed even in diabetic subjects with good metabolic control (Verges 2009).

Periodontitis may have an influence on glycemic control and therefore enhance the development of other diabetic complications (Donahue & Wu 2001, Nishimura *et al.* 2003). Some evidence exists that anti-infective treatment of periodontal disease has a modest but beneficial effect on glycemic control of diabetic patients, but so far the available data are extremely limited (Taylor & Borgnakke 2008, Simpson *et al.* 2010, Chen *et al.* 2012). However, it cannot be ruled out that the so-called 'perio-systemic' relationship may be two-directional, meaning increased susceptibility to gingivitis and periodontitis under hyperglycemia but, on the contrary, improved glycemic control after elimination of periodontal infection.

3 Aims of the study

The main purpose of this study was to explore the association between systemic inflammatory status and periodontal inflammation and tissue destruction. The periodontal variables used were bleeding on probing, probing pocket depth, and periodontal attachment loss, and the studied systemic inflammatory markers/mediators were IL-6, TNF-α, IL-10, and HDL. More specifically, the purpose was to study

- the association between serum IL-6 and periodontal inflammation in T1DM subjects in a longitudinal setting before and after periodontal therapy,
- the association between serum TNF-α and IL-10 and periodontal inflammation and tissue destruction in a cross-sectional setting in subjects with moderate to severe periodontitis,
- the association between serum HDL level and periodontal inflammation in T1DM subjects in a cross-sectional setting.

In addition, the role of MMP-8 in non-inflamed gingival sites as a marker of an individual's overall susceptibility to periodontal inflammation and tissue destruction was studied.

4 Material and methods

4.1 Subjects

The study population of this work consisted of three groups of subjects of Caucasian origin: 1) subjects with chronic periodontitis (n = 61), 2) subjects with T1DM (n = 80) and 3) periodontally healthy control subjects (n = 30). Since all data were not available for all subjects, the number of subjects in the periodontitis group varies in Papers II and IV.

Table 1. Characteristics of the study groups

Study group	n	Age mean ± SD	Females n (%)	Smokers n (%)
Periodontitis group				
Paper II	61	46.4 ± 13.3	39 (63.9)	36 (59.0)
Paper IV	48	44.6 ± 11.3	32 (66.7)	28 (58.3)
Diabetes group				
Baseline				
Papers I and III	80	38.6 ± 12.3	46 (57.5)	24 (30.0)
After therapy				
Paper I	58	39.5 ± 12.6	34 (58.6)	14 (24.1)
Control group				
Paper II	30	41.9 ± 12.7	19 (63.3)	10 (33.3)

All the subjects were examined by the same periodontal specialist (T.R.) at the Specialist Dental Health Care Unit, City of Oulu, Finland. Informed consent was obtained from all subjects and the study protocol was accepted by the Ethical Committee of Oulu University Hospital, Oulu, Finland.

Subjects needing prophylactic antibiotic medication in association with periodontal probing and periodontal therapy as well as those with immunosuppressive medication or antibiotics during the past four months were excluded from the study.

The subjects in the periodontitis group (Papers II, IV) had moderate to severe chronic periodontitis. They were principally periodontally untreated and were referred to periodontal specialist therapy at the Specialist Dental Health Care Unit, City of Oulu, Finland. Subjects with rheumatoid arthritis, diabetes mellitus, and asthma were excluded from this group.

In the diabetic group (Papers I, III), all the subjects had type 1 diabetes mellitus (T1DM) and they were mainly recruited from the Diabetes Clinic of Oulu Health Centre. A few patients were recruited from The Clinic of Internal Medicine, Oulu University Hospital, Oulu, Finland. Patient records were used to retrieve data regarding diabetic state and the duration of the disease.

The control group (Paper II) consisted of systemically healthy subjects with the least amount of periodontal inflammation. They were volunteer subjects from among patients of the Dental Health Center of the City of Oulu and the university staff and students, who were informed of the study. In addition, the subjects were matched by age and gender with the periodontitis group.

4.2 Clinical periodontal examination and periodontal therapy

Clinical measurements were performed on four surfaces (mesiobuccal, midbuccal, distobuccal, and midlingual) of each tooth, excluding third molars. After drying with air, presence of visible plaque was assessed corresponding to scores 2 and 3 of the (Silness & Löe 1964) plaque index. A ball-pointed periodontal probe with 2-mm graduations was used to measure probing/pocket depth (PD) from the gingival margin to the base of the crevice/pocket. A positive score for bleeding on probing (BOP) was registered if a site presented bleeding 20–30 seconds after probing. Periodontal attachment level (AL) from the cementoenamel junction to the base of the crevice/pocket was also registered. The extent of periodontal inflammation/disease was expressed as numbers or percentages of periodontally affected sites (Table 2).

Table 2. Reported periodontal parameters of the study groups.

Study group	BOP	PD ≥ 4 mm	BOP and PD ≥ 4 mm	AL ≥ 4 mm	Number of teeth
Periodontitis group					
Paper II*	81.3 ± 22.1	50.6 ± 24.8		36.3 ± 22.2	25.4 ± 3.3
Paper IV†	78.7 ± 19.1	45.7 ± 20.3		40.3 ± 25.4	25.5 ± 3.4
Diabetes group*					
Baseline	69.3 ± 18.1		23.1 ± 18.5		25.7 ± 4.1
After therapy	14.9 ± 12.9		1.0 ± 2.1		26.0 ± 3.8
Control group*	15.5 ± 11.1	1.0 ± 2.4		0.6 ± 1.4	27.4 ± 1.0

*Individual numbers of affected sites ± SD

†Individual percentages of affected sites ± SD

BOP, bleeding on probing; PD, pocket depth; AL, attachment level

Anti-infective periodontal therapy, including oral hygiene education, scaling and root planing, and periodontal surgery whenever indicated, was performed on the subjects in the diabetic group. A clinical follow-up examination was performed approximately eight weeks after completion of the therapy (Paper I).

Data concerning smoking habits were obtained by interviewing the subjects in conjunction with the clinical examination and the subjects were categorized as smokers and non-smokers. The subjects' body mass index (BMI, weight divided by the square of height, kg/m^2) and age were also recorded.

4.3 Sampling of gingival crevicular fluid (GCF) and serum

The selection of four healthy and four diseased sites for GCF collection was based on visual inspection for clinical signs of inflammation. Shallow crevices include sites with PD $\leq$ 3 mm, and the subjects were divided into subgroups according to the presence of BOP: in subgroup A (n = 30), at most one site of the four shallow sites selected for sampling presented bleeding on probing, and in subgroup B (n = 21), there was no bleeding on probing at the sampled sites. All the diseased sites presented bleeding on probing and 90% were $\geq$ 4mm deep.

The area of GCF collection was isolated using cotton rolls, supragingival plaque was removed, and a paper strip (Periopaper IDE Interstate, Amityville, NJ) was placed at the orifice of the crevice/pocket for thirty seconds. After that the strips were placed in 50 µl of 0.9% saline (PBS). After one hour in ice the GCF samples were centrifuged at 9220 x g for 15 minutes and stored at -70°C until analyzed. A venous blood sample was drawn from each subject on the day of the examination and serum samples were stored at -70°C until assayed.

4.4 Laboratory analyses

The serum IL-6, IL-10, and TNF-α levels were analyzed using commercially available enzyme-linked immunosorbent assays (ELISA) (Quantikine® Human IL-6, R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany; Quantikine® Human IL-10, Quantikine® Human TNF-α, R&D Systems Europe, Ltd, Abingdon, UK). While the actual levels of IL-6 (range 0.4–6.2 pg/ml at the baseline, 0.4–8.2 pg/ml after therapy) and IL-10 (range 6.6–74.3 pg/ml) were expressed as pg/ml, the levels of TNF-α (range 0.0–62.5 U/l) were expressed as units per liter (U/l), corresponding to OD 450–540 nm x 10E3. Three subjects (5%) in the periodontitis group and six subjects (20%) in the control group

presented TNF-α levels that were under the detection limit (DL) (0.5 U/l), and the DL/2 method (Uh *et al.* 2008) was used to substitute these values. The individual TNF-α/IL-10 ratios were calculated by dividing the actual serum TNF-α levels by the IL-10 levels.

The glycosylated hemoglobin value (HbA1c, %) of each subject in the diabetes group was analyzed after sampling using a latex immunoturbidimetric method (Siemens Medical Solutions Diagnostics, Tarrytown, NY 10591–5097 USA). The usCRP (ultrasensitive CRP, mg/l) analyses were made using an Innotrac Aio! analyzer (Innotrac Diagnostics Oy, Turku, Finland), a fully automated random-access immunoanalyzer that utilizes a unique all-in-one (Aio!) dry-reagent concept and time-resolved fluorometric detection (Hedberg *et al.* 2004). The 10% CV limit and the detection limit for the Innotrac Aio! usCRP assay are 0.15 mg/l and 0.003 mg/l, respectively.

The serum levels of high-density (HDL) and low-density (LDL) cholesterols were measured using direct enzymatic methods implemented in the ADVIA Chemistry 2400 system (Siemens Medical Solutions Diagnostics, Tarrytown, NY 10591–5097 USA). Serum triglyceride level was determined using enzymatic and photometric methods of the same system.

Before the analysis, the GCF samples collected from four shallow and four diseased sites were separately pooled together (50 µl of 0.9% saline (PBS) x 4). Concentration of GCF MMP-8 was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (Quantikine®, R&D Systems Inc., Minneapolis, USA) according to the manufacturer's instructions. The MMP-8 concentration was analyzed as ng/ml of the sample (200 µl 0.9% saline and the GCF absorbed into the strips).

4.5 IL-6-174, IL-10-1082, and TNF-α-308 polymorphisms

Cytokine gene polymorphisms of *IL-6*-174 and *TNF-α*-308 were tested using a PCR (polymerase chain reaction) with a RFLP (restriction fragment length polymorphism) (Fishman *et al.* 1998, Ozen *et al.* 2002). *IL-10*-1082 was tested using a simple bidirectional allele-specific PCR method developed by our own group (Karhukorpi & Karttunen 2001). Genetic analyses were performed blind to clinical status. Earlier tested DNA specimens from the patients, representing both alleles for each single-nucleotide polymorphism (SNP), were used in each test series as control samples for the purpose of confirming the performance of the method (PCR, restriction enzyme). Aproximately 10–20% of the patient samples

in each SNP, including the above-mentioned controls, were retested for SNP. No error was found in retesting (error rate 0), and therefore no more retesting was considered necessary. Hidden duplicates were not used.

Deviations from Hardy-Weinberg equilibrium were defined using Pearson's goodness-of-fit method between the observed and expected frequencies in a reference group consisting of university staff and students of Caucasian origin (122 females and 56 males aged 39.4 ± 13.4 years) and representing the normal population of the area. They were informed of the study and volunteered to give their blood samples to be used as reference samples (Karhukorpi *et al.* 2002). The genotype distributions of the studied single nucleotide polymorphisms were in Hardy-Weinberg equilibrium.

The AG/GG genotype of *IL-10*-1082 is associated with higher secretion of the regulatory cytokine IL-10 than is the AA genotype (Turner *et al.* 1997). The genotypes of *TNF-α*-308 that contain A allele(s) are known to be associated with higher TNF-α transcription (Kroeger *et al.* 1997) and secretion (McGuire *et al.* 1994). Furthermore, the G-containing genotype of *IL-6*-174 is known to be associated with higher IL-6 secretion (Hulkkonen *et al.* 2001). Based on the above the subjects were categorized into the following genotype categories: IL-10-1082 AG/GG vs AA, TNF-α-308 AG/AA vs GG, IL-6-174 CG/CC vs GG.

4.6 Statistical methods

4.6.1 Post-hoc sample size evaluation using Cohen's (1988) power analysis approach

To evaluate the sample size of the T1DM group post-hoc, linear regression analysis of the association between serum HDL (the main explanatory variable) and the extent of bleeding and PD ≥ 4 mm (the outcome) was utilized (Paper III, Table 6). After adjustments were made for HbA1c, age, smoking, gender, number of sites with plaque, and number of measured sites, the R^2 of the model was 0.419. The calculation indicated that to obtain a power level of 0.90 (with α = 0.05), a minimum of 34 T1DM subjects would be needed in future studies (Soper 2012).

A corresponding calculation for the size of the control and the periodontitis group was performed utilizing the regression model of the association between the extent of sites with PD ≥ 4 mm (the main explanatory variable) and serum IL-10 level (the outcome variable) (Paper II, Table 3). After adjusting for age, gender,

smoking, and BMI, the R^2 of that model was 0.255. To obtain a power level of 0.90 (with a = 0.05), altogether 54 control and periodontitis subjects would be needed in future studies.

4.6.2 Statistical methods used

All statistical analyses were carried out with the aid of statistical software (SPSS, version 16.0; SPSS Inc., Chicago, IL, USA), and the level of statistical significance was set at < 5% ($p < 0.05$).

As regards periodontal variables, the analyses were based on subject-level data using individual percentages or numbers of periodontally affected sites.

Both Student's t-test and the non-parametric Mann-Whitney test were used to study the significances of differences in mean values.

Pearsons's and Spearman's rank correlation analyses were used in assessing the unadjusted associations between the extent of periodontal inflammation and tissue destruction and the levels of inflammatory markers/mediators. In order to control for confounding/modifying factors, multivariate models (linear or negative binomial regression) and stratification of the study populations (by the amount of plaque, smoking, and BMI) were used.

5 Results

5.1 Serum interleukin-6 may modulate periodontal inflammation in type 1 diabetic subjects (Paper I)

In this study the subjects were periodontally treated after the baseline examination and re-examined eight weeks after completion of the therapy. Anti-infective therapy was successful, as indicated by the reduction in the mean number of sites with BOP and bleeding and PD ≥ 4 mm per subject (from 69.3 ± 18.1 to 14.9 ± 12.9, from 23.1 ± 18.5 to 1.0 ± 2.1, respectively). Despite the successful therapy, no essential changes were observed in the mean values of serum IL-6 and HbA1c from the baseline (1.7 ± 1.0 and 8.5 ± 1.4, respectively) to the follow-up examination (1.6 ± 1.1 and 8.4 ± 1.4, respectively). Statistically significant associations were found between serum IL-6 level and the number of sites with bleeding and PD ≥ 4 mm in both subjects with BMI ≤ 26 kg/m2 and non-smokers (Table 3, Models I and II). After periodontal therapy, a statistically significant association was found between serum IL-6 level and the number of sites with bleeding and PD ≥ 4 mm in subjects with BMI ≤ 26 kg/m2 (p = 0.043), and this association was of borderline significance in non-smokers (p = 0.064) (Table 3, Models III and IV). The adjusted associations between serum IL-6 level and the number of sites with BOP and bleeding and PD ≥ 4 mm were not statistically significant in subjects with BMI > 26 kg/m2 (n = 14) and in smokers (n = 14) after therapy (data not shown).

Table 3. Adjusted associations between the number of bleeding sites (BOP) and sites with bleeding and PD ≥ 4 mm (the outcome variables) and serum IL-6 level at the baseline and after therapy in subjects with BMI ≤ 26 kg/m2 and in non-smokers.

Outcome variable	B	95% CI for B	p
Baseline			
I Subjects with BMI ≤ 26 kg/m^2 (n = 58)			
BOP*			
IL-6	3.029	–0.553 to 6.612	0.096
HbA1c	4.032	1.515 to 6.549	0.002
Smoking‡	–6.383	–14.429 to 1.662	0.117
Bleeding and PD ≥ 4 mm†			
IL-6	0.327	0.075 to 0.578	0.011
HbA1c	0.274	0.087 to 0.460	0.004
Smoking‡	–0.653	–1.245 to 0.061	0.031

Outcome variable	B	95% CI for B	p
II Non-smokers (n = 56)			
BOP*			
IL-6	1.021	–2.686 to 4.728	0.582
HbA1c	3.998	1.181 to 6.815	0.006
Bleeding and PD ≥ 4 mm[†]			
IL-6	0.288	0.040 to 0.536	0.023
HbA1c	0.218	0.011 to 0.425	0.039
After therapy			
III Subjects with BMI ≤ 26 kg/m^2 (n = 58)			
BOP[†]			
IL-6	0.287	–0.069 to 0.644	0.114
HbA1c	0.078	–0.159 to 0.315	0.518
Smoking[‡]	–0.210	–0.968 to 0.548	0.588
Bleeding and PD ≥ 4 mm[†]			
IL-6	0.550	0.016 to 1.084	0.043
HbA1c	–0.052	–0.454 to 0.350	0.800
Smoking[‡]	0.308	–0.911 to 1.527	0.621
IV Non-smokers (n = 56)			
BOP[†]			
IL-6	0.142	–0.146 to 0.429	0.335
HbA1c	0.015	–0.214 to 0.244	0.896
Bleeding and PD ≥ 4 mm[†]			
IL-6	0.402	–0.023 to 0.827	0.064
HbA1c	0.040	–0.320 to 0.401	0.827

*Linear and [†]negative binomial regression models.

Models I, III; adjusted for age, gender, smoking, HbA1c, serum HDL, plaque, and number of sites.

Models II, IV; adjusted for age, gender, HbA1c, BMI, serum HDL, plaque, and number of sites.

[‡]Smoking as a categorized variable; non-smokers *versus* smokers (reference).

Boldface denotes statistical significance.

Bleeding and PD ≥ 4 mm, bleeding pocket depth ≥ 4 mm; BMI, body mass index; HbA1c, glycosylated hemoglobin; IL, interleukin.

A decrease (≥ 0.2 pg/ml) in the level of serum IL-6 between the baseline and the follow-up examination was observed in 23 (39.7%) subjects, an increase (≥ 0.2 pg/ml) in 17 (29.3%) subjects, and a stable level in 18 (31.0%) subjects. Periodontal healing, described as a reduction in sites with bleeding and PD ≥ 4 mm, was similar in subjects with a decreased, an increased, or a stable level of serum IL-6 (Figure 1, A+B).

Fig1.

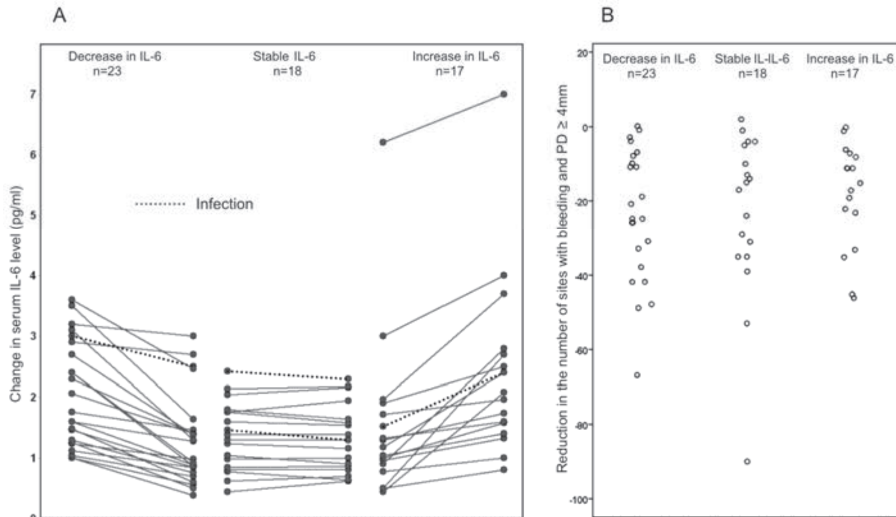

Fig 2 A. Lines on the left represent subjects with a decrease in serum IL-6 level (≥ 0.2 pg/ml) and) the lines on the right represent subjects with an increase (≥ 0.2 pg/ml) in serum IL-6 level after therapy. The lines in the middle present subjects with stable IL-6 level (change < 0.2 pg/ml). Dashed lines indicate subjects with chronic infections, other than gingivitis or periodontitis. Fig 2 B. Healing of periodontal inflammation indicated as a reduction in the number of sites with bleeding and PD ≥ 4 mm in subjects presenting an increased, stable, or decreased serum IL-6 level between the baseline and the follow-up examination.

The higher the number of sites with residual inflammation (sites with bleeding and PD ≥ 4 mm after therapy), the higher the actual mean levels of serum IL-6 observed (Table 4).

Table 4. Mean serum IL-6 levels (± S.D., pg/ml) according to the number of sites with bleeding and PD ≥ 4 mm after periodontal therapy

Subjects	BMI ≤ 26 kg/m^2		Non-smokers	
	n	IL-6 (pg/ml)	n	IL-6 (pg/ml)
All subjects	44	1.6 ± 1.1	44	1.6 ± 1.1
Subjects with				
No sites with bleeding and PD ≥ 4 mm	29	1.2 ± 0.6	27	1.3 ± 0.6
≥ 1 sites with bleeding and PD ≥ 4 mm	15	2.2 ± 1.6	17	2.0 ± 1.5
≥ 2 sites with bleeding and PD ≥ 4 mm	8	2.9 ± 1.9	10	2.3 ± 1.8
≥ 3 sites with bleeding and PD ≥ 4 mm	5	3.2 ± 2.3	5	2.8 ± 2.4

Bleeding and PD ≥ 4 mm, bleeding pocket depth ≥ 4mm; BMI, body mass index; IL, interleukin.

A statistically significant association ($p = 0.003$) was found between the reduction in the number of sites with bleeding and PD ≥ 4 mm and the after-therapy level of serum IL-6, indicating that subjects with high serum IL-6 presented poorer periodontal healing (Table 5).

Table 5. Adjusted associations between periodontal healing (the outcome variable) and serum IL-6 level (pg/ml) after periodontal therapy using linear regression analysis.

Explanatory variable	B	95% CI for B	p-value
IL-6 after therapy (pg/ml)	–0.973	–1.599 to 0.348	0.003
IL-6 at the baseline (pg/ml)	0.381	–0.288 to 1.050	0.258
Number of sites with bleeding and PD ≥ 4 mm at the baseline	0.951	0.922 to 0.980	< 0.001
Reduction in the number of sites with plaque	0.028	0.002 to 0.055	0.039
Smoking	–0.800	–1.920 to 0.320	0.158
BMI	0.014	–0.141 to 0.168	0.860

The outcome variable: the number of sites with bleeding and PD ≥ 4 mm after therapy deducted from the number of sites at the baseline (range: –2 to 90).
Smoking as a categorized variable; non-smokers *versus* smokers (reference).
Bleeding and PD ≥ 4 mm, bleeding pocket depth ≥ 4 mm; BMI, body mass index; IL, interleukin.

5.2 Associations between the extent of periodontal disease and serum levels of interleukin (IL)-10 and tumor necrosis factor (TNF)-α and their ratio (Paper II)

To study dose dependency between serum IL-10 and TNF-α and their ratio and the extent of periodontal disease, the subjects with chronic periodontitis were

categorized into tertiles according to the extent of sites with BOP, PD ≥ 4 mm, and AL ≥ 4 mm (I-III). The reference category was comprised of the control subjects. The subjects in the periodontitis group presented lower serum IL-10 and higher serum TNF-α mean levels than the control subjects. Furthermore, the TNF-α/IL-10 ratio was approximately three times lower in the control subjects than in the periodontitis subjects (Table 6).

Table 6. The mean serum IL-10 (pg/ml) and TNF-α (U/l) levels and TNF-α/IL-10 ratios (the actual serum TNF-α levels divided by the IL-10 levels) in the control and periodontitis subjects.

Group/subjects	n	IL-10 (95% CI)	TNF-α (95% CI)	TNFα/IL-10 (95% CI)
Control group	30	17.4 (12.9–21.9)	9.2 (5.9–12.4)	0.6 (0.4–0.8)
Periodontitis group				
BOP				
Tertile I (1–67, 55.0 ± 13.6)	20	11.6 (10.1–13.1)	23.3 (15.4–31.2)	2.0 (1.3–2.8)
Tertile II (68–95, 85.0 ± 8.5)	20	9.9 (8.9–11.0)	15.8 (9.1–22.6)	1.5 (1.0–2.0)
Tertile III (> 95, 102.8 ± 4.6)	21	11.5 (8.8–14.3)	20.3 (12.0–28.6)	1.9 (1.1–2.8)
PD ≥ 4 mm				
Tertile I (1–36, 24.3 ± 9.4)	20	11.3 (9.8–12.9)	21.5 (14.0–29.1)	1.9 (1.2–2.5)
Tertile II (37–58, 48.5 ± 6.7)	21	10.2 (9.2–11.2)	18.8 (11.5–26.2)	1.8 (1.2–2.4)
Tertile III (> 58, 78.2 ± 15.0)	20	11.6 (8.7–14.5)	19.1 (10.6–27.7)	1.8 (0.9–2.6)
AL ≥ 4 mm				
Tertile I (1–23, 12.5 ± 7.1)	20	11.1 (9.9–12.4)	20.7 (12.9–28.5)	1.8 (1.2–2.4)
Tertile II (24–43, 35.9 ± 6.0)	21	9.7 (8.6–10.7)	16.8 (10.2–23.5)	1.7 (1.0–2.3)
Tertile III (> 43, 60.2 ± 15.4)	20	12.4 (9.5–15.3)	22.1 (13.3–30.9)	2.0 (1.1–2.8)

The subjects in the periodontitis group were divided into tertiles according to the number of sites with bleeding on probing (BOP), pocket depth ≥ 4 mm (PD ≥ 4 mm), and attachment level ≥ 4 mm (AL ≥ 4 mm). The numbers in the parentheses in the left column indicate ranges and the mean numbers (± SD) of periodontally affected sites. TNF, tumor necrosis factor; IL, interleukin.

The adjusted associations between serum IL-10 level and the extent of periodontal disease, using the control group as a reference, indicated that the subjects in the control group had significantly higher levels of IL-10 than the subjects in any of the BOP, PD ≥ 4 mm, or AL ≥ 4 mm tertiles (Table 7, on the left). Overall, no consistent association between serum IL-10 and periodontal variables were found inside the periodontitits group (Table 7, on the right).

Table 7. Associations between IL-10 serum levels and the extent of BOP, PD ≥ 4 mm, and AL ≥ 4 mm (numbers of affected sites) analyzed using a multivariate linear regression model adjusted for age, gender, smoking, and BMI.

The main explanatory variable/group		n	B	95% CI for B	p-value	B (95% CI for B) p-value	B (95% CI for B) p-value
BOP	Control subjects	30		Reference			
	Periodontitis group						
	Tertile I	20	–0.35	–0.58 to –0.13	0.003	Tertile II versus I	Tertile III versus II
	Tertile II	20	–0.47	–0.67 to –0.26	<0.001	–0.29 (–0.48 to –0.10)	–0.11 (–0.30 to –0.08)
	Tertile III	21	–0.38	–0.59 to –0.17	0.001	p = 0.003	p = 0.265
PD ≥ 4 mm	Control subjects	30		Reference			
	Periodontitis group						
	Tertile I	20	–0.38	–0.60 to –0.15	0.001	Tertile II versus I	Tertile III versus II
	Tertile II	21	–0.44	–0.65 to –0.24	<0.001	–0.26 (–0.44 to –0.07)	–0.10 (–0.29–0.09)
	Tertile III	20	–0.38	–0.59 to –0.17	0.001	p = 0.007	p = 0.285
AL ≥ 4 mm	Control subjects	30		Reference			
	Periodontitis group						
	Tertile I	20	–0.37	–0.57 to –0.17	0.001	Tertile II versus I	Tertile III versus II
	Tertile II	21	–0.50	–0.70 to –0.29	<0.001	–0.31 (0.50 to –0.13)	–0.03 (–0.22–0.15)
	Tertile III	20	–0.32	–0.54 to –0.11	0.004	p = 0.001	p = 0.727

On the left: estimates of the analyses using the control group as a reference; on the right: estimates using adjacent categories.

The adjusted associations between serum TNF-α level and the extent of periodontal disease indicated that the control subjects had significantly lower serum TNF-α levels than the subjects in each of the BOP, PD ≥ 4 mm, and AL ≥ 4 mm tertiles (I–III) (Table 8).

Table 8. Associations between TNF-α serum levels and the extent of BOP, PD ≥ 4 mm, and AL ≥ 4 mm (numbers of affected sites) analyzed using a multivariate linear regression model adjusted for age, gender, smoking, and BMI.

The main explanatory variable/group	n	B	95% CI for B	p-value
BOP				
Control group	30		Reference	
Periodontitis group				
Tertile I	20	1.51	0.68–2.35	0.001
Tertile II	20	0.80	0.03–1.57	0.042
Tertile III	21	1.11	0.33–1.90	0.006
PD ≥ 4 mm				
Control group	30		Reference	
Periodontitis group				
Tertile I	20	1.21	0.37–2.10	0.005
Tertile II	21	1.19	0.42–2.00	0.003
Tertile III	20	0.95	0.16–1.75	0.019
AL ≥ 4 mm				
Control group	30		Reference	
Periodontitis group				
Tertile I	20	1.11	0.35–1.88	0.005
Tertile II	21	0.86	0.09–1.63	0.029
Tertile III	20	1.42	0.61–2.23	0.001

Estimates of analyses using the control group as a reference.

IL, interleukin; AL, attachment level; PD, pocket depth; BOP, bleeding on probing; BMI, body mass index.

Overall, no statistically significant differences in TNF-α levels were found between adjacent periodontitis categories (data not shown). Furthermore, the associations between serum TNF-α/IL-10 ratio (the outcome) and the extent of periodontal disease indicated significantly higher ratios in all BOP, PD ≥ 4 mm, and AL ≥ 4 mm tertiles (I–III) when compared with the ratio in the control group (data not shown).

5.3 Serum high-density lipoprotein cholesterol level associated with the extent of periodontal inflammation in type 1 diabetic subjects (Paper III)

A statistically significant association was found between the number of sites with bleeding and PD ≥ 4 mm (the outcome variable) and serum HDL level (B = -9.72, p = 0.022) in a linear regression model (Table 9).

Table 9. Adjusted associations between the number of sites with bleeding and PD ≥ 4 mm (the outcome variable) and serum HDL (mmol/l).

Explanatory variable	B	95 CI for B	p-value
HDL (mmol/l)	–9.72	–18.0 to –1.44	0.022
HbA1c (%)	2.90	0.45 to 5.36	0.021
Age	0.34	0.02 to 0.66	0.037
Male gender	–1.71	–9.12 to 5.70	0.647
Non-smoker	–10.97	–18.51 to –3.44	0.005
Number of sites with plaque	0.40	0.24 to 0.55	< 0.001
Number of measured sites	0.16	–0.09 to 0.40	0.212

Linear regression analysis:

HbA1c (%), age, number of sites with plaque, and number of measured sites as continuous variables.

Gender and smoking as categorized variables; males versus females (reference) and non-smokers versus smokers (reference).

PD, pocket depth; HDL, high-density lipoprotein cholesterol; HbA1c, glycosylated hemoglobin

The association was even stronger after including the IL-6-174 genotype in the model (B = 12.76, p = 0.002) (Table 10), but no statistically significant interaction was found between the IL-6-174 genotype and serum HDL in a separate model (data not shown).

Table 10. Adjusted associations between the number of sites with bleeding and PD ≥ 4 mm (the outcome variable) and serum HDL (mmol/l) and IL-6-174 genotype (explanatory variables).

Explanatory variable	B	95% CI for B	p-value
HDL (mmol/l)	–12.76	–20.72 to –4.79	0.002
GG genotype of *IL-6*-174	13.59	5.46 to 21.71	0.001
HbA1c (%)	2.45	0.14 to 4.77	0.038
Age	0.26	–0.04 to 0.56	0.086
Male gender	–5.12	–12.35 to 2.11	0.162
Non-smoker	–11.51	–18.58 to –4.45	0.002
Number of sites with plaque	0.38	0.23 to 0.52	<0.001
Number of measured sites	0.15	–0.09 to 0.38	0.213

Linear regression analysis:
HbA1c (%), age, number of sites with plaque, and number of measured sites as continuous variables.
IL-6^{-174} genotype, gender, and smoking as categorized variables; GG *versus* GC+CC (reference), males *versus* females (reference) and non-smokers *versus* smokers (reference).
PD, pocket depth; HDL, high-density lipoprotein cholesterol; IL, interleukin; HbA1c, glycosylated hemoglobin

The previous analysis was also done among non-smokers, and the association between the number of sites with bleeding and PD ≥ 4 mm (the outcome variable) and serum HDL level remained evident (B = 9.86, p = 0.048, data not shown).

In order to study the linearity of the association between periodontal inflammation and serum HDL level, the subjects were divided into tertiles according to ascending serum level of HDL (mmol/l) (Table 11).

Table 11. Mean percentages of sites with bleeding and PD ≥ 4 mm and numbers of smokers in serum HDL tertiles.

HDL tertiles limits (mmol/l)	n	Smokers	Bleeding and PD ≥ 4 mm (%) (mean, 95% CI)
Tertile I			
<1.35	27	7	31.4 (21.6 to 41.2)
Tertile II			
1.35–1.69	27	10	20.3 (13.8 to 26.8)
Tertile III			
> 1.69	26	7	19.0 (12.8 to 25.2)

PD, pocket depth; HDL, high-density lipoprotein cholesterol

Confirming the unadjusted associations between periodontal inflammation and serum HDL level (Table 11), the adjusted associations also indicated that the

subjects in HDL tertiles II and III presented fewer sites with bleeding and PD ≥ 4 mm when compared with the subjects in tertile I (reference) ($p = 0.046$ and $p = 0.002$, respectively). No such difference was observed between tertiles II and III ($p = 0.272$) (Table 12).

Table 12. Associations between the number of sites with bleeding and PD ≥ 4 mm (the outcome variable) and serum HDL (classified into tertiles).

HDL tertiles	n	B	95% CI for B	p-value
Tertile I	27		Reference	
Tertile II	27	–8.13	–16.09 to –0.17	0.046
Tertile III	26	–12.74	–20.57 to –4.91	0.002
Tertile II	27		Reference	
Tertile III	26	–4.61	–12.90 to 3.69	0.272

Linear regression analysis adjusted for HbA1c (%), age, gender, smoking, number of sites with plaque, and number of measured sites.

Tertile limits as presented in Table 14.

PD, pocket depth; HDL, high-density lipoprotein cholesterol; HbA1c, glycosylated hemoglobin

5.4 Association between matrix metalloproteinase (MMP)-8 concentration in shallow crevices and the extent of attachment loss in chronic periodontitis (Paper IV)

The mean concentration of MMP-8 in shallow sites was 11.8 ng/ml and in diseased sites, 150.1 ng/ml (Table 13).

Table 13. Characteristics of the subjects.

Subject characteristic	All subjects (n = 48)	Group A (n = 30)	Group B (n = 21)
Periodontal parameters*			
Mean ± SD			
Plaque	24.7 ± 19.7	21.1±20.4	23.4 ± 22.4
BOP	78.7 ± 19.1	70.9 ± 19.2	72.0 ± 16.3
PD ≥ 4 mm	45.7 ± 20.3	37.8 ± 18.4	37.7 ± 17.9
PD ≥ 6 mm	12.2 ± 14.9	9.6 ± 12.7	10.1 ± 12.8
AL ≥ 4 mm	40.3 ± 25.4	34.8 ± 22.8	35.9 ± 24.1
AL ≥ 6 mm	13.3 ± 15.4	10.8 ± 12.3	11.8 ± 13.6
MMP-8 concentration (ng/ml)			
Mean ± SD			
Shallow sites	11.8 ± 12.8	11.4 ± 12.6	12.4 ± 14.3

Subject characteristic	All subjects (n = 48)	Group A (n = 30)	Group B (n = 21)
Diseased sites	150.1 ± 91.8	150.1 ± 97.4	149.0 ± 95.9
Serum	7.6 ± 4.6	7.6 ± 4.9	7.8 ± 5.6

The subjects in Group A presented 0-1 sites (out of four sampled sites) with bleeding on probing (BOP) and the subjects in Group B presented no bleeding in the sampled sites.
*Individual percentages of affected sites
BOP, bleeding on probing; PD, pocket depth; AL, attachment level; MMP-8, matrix metalloproteinase-8.

The unadjusted associations between MMP-8 level in shallow crevices and the subject-level extent of periodontal disease (PD ≥ 4 mm, AL ≥ 4 mm, and AL ≥ 6 mm) were statistically significant. No such associations were found in diseased sites (Table 14).

Table 14. Correlations between the extent of periodontal disease (% of sites per subjects) and MMP-8 concentration in GCF from shallow crevices and diseased sites of all subjects (n = 48)

Sampling site	BOP	PD ≥ 4 mm	PD ≥ 6 mm	AL ≥ 4 mm	AL ≥ 6 mm
Shallow crevices	0.275	0.368	0.259	0.435	0.558
	p = 0.058	p = 0.010	p = 0.075	p = 0.002	p <0.001
Diseased sites	0.153	0.171	0.256	–0.045	–0.066
	p = 0.299	p = 0.244	p = 0.079	p = 0.760	p = 0.658

BOP, bleeding on probing; PD, pocket depth; AL, attachment level; GCF, gingival crevicular fluid; MMP-8, matrix metalloproteinase-8.

The interaction terms between MMP-8 concentration and both smoking and plaque scores were studied. Due to the statistically significant interaction between MMP-8 and plaque, the sample was stratified according to plaque score (≤ 12.5% vs. > 12.5%), and the associations between MMP-8 concentration and the extent of AL ≥ 4 mm and AL ≥ 6 mm (the outcome variables) were studied in the stratified samples. After adjusting for smoking, a statistically significant association was found between MMP-8 concentration in shallow crevices and the extent of both AL ≥ 4 mm ($p = 0.028$) and AL ≥ 6 mm ($p < 0.001$) in subjects with high plaque scores. No such associations were found in subjects with low plaque scores (Table 15). No interaction was found between MMP-8 concentration and smoking.

Table 15. Smoking-adjusted associations between MMP-8 (ng/ml) in GCF from shallow sites and extent of AL ≥ 4 mm and AL ≥ 6 mm in subjects with low (≤ 12.5%, n = 12) and high (> 12.5%, n = 36) individual plaque scores

Outcome variable	B	95% CI for B	p-value
Low plaque scores			
AL ≥ 4 mm			
MMP-8 (mmol/l)	1.4	–1.7 to 4.6	0.323
Smoking	–0.03	–28.0 to 28.0	0.998
AL ≥ 6 mm			
MMP-8 (mmol/l)	1.0	–0.1 to 2.2	0.068
Smoking	6.3	–3.9 to 16.5	0.193
High plaque scores			
AL ≥ 4 mm			
MMP-8 (mmol/l)	0.7	0.1 to 1.3	0.028
Smoking	–14.3	–32.5 to 3.9	0.119
AL ≥ 6 mm			
MMP-8 (mmol/l)	0.7	0.4 to 1.1	<0.001
Smoking	–0.5	–11.1 to 10.2	0.930

Smoking, non-smokers versus smokers.

AL, attachment level, mean percentage of affected sites per subjects; MMP-8, matrix metalloproteinase-8.

Three different cut-off points of the percentage of sites with plaque were used: the lowest versus the three highest quartiles (cut-off point 12.5%), the lowest versus the two highest tertiles (cut-off point 15.0%) and the median (cut-off point 20.0%). Analyses using various cut-off points yielded similar results.

6 Discussion

6.1 The main findings and their significance

The principal finding of the present study was the evident bidirectional association between periodontal inflammation and systemic inflammatory status. More specifically, in subjects with T1DM, serum IL-6 level correlated significantly with the number of sites with inflamed periodontal pockets both before periodontal therapy (the baseline) and after elimination of the bacterial burden using anti-infective periodontal therapies. In addition, periodontal healing turned out to be poorer in subjects with a high after-therapy IL-6 level than in those with a low level. On the other hand, elimination of periodontal inflammation did not consistently result in decreased serum IL-6 levels. The serum TNF-α/IL-10 ratio was three times higher in chronic periodontitis patients when compared with periodontally healthy controls, indicating a stronger systemic pro-inflammatory status in the periodontitis group. A statistically significant negative association was found between serum HDL and periodontal inflammation in subjects with T1DM. Subjects with a serum HDL level < 1.35 mmol/l presented a significantly higher extent of periodontal inflammation when compared with subjects with a serum HDL level ≥ 1.35 mmol/l. The GCF level of MMP-8, a marker of local inflammatory response, in non-inflamed crevices was significantly associated with the overall extent of periodontal attachment loss in subjects with chronic periodontitis.

In summary, the above results support the proposed association between periodontal inflammation and systemic inflammatory status and the hypothesis that subjects with chronic periodontitis may present a stronger systemic pro-inflammatory state than periodontally healthy subjects. In this respect, chronic periodontitis seems to be identical to several other inflammatory/infectious conditions, like rheumatoid arthritis (Kokkonen *et al.* 2010), myocardial infarction (Karpinski *et al.* 2009), inflammatory bowel disease (Szkaradkiewicz *et al.* 2009), and alcoholic liver disease (McClain *et al.* 2004), in which a predominance of pro-inflammatory processes is observed.

Periodontitis patients have been reported to present elevated levels of serum inflammatory markers and bacterial products (e.g. LPS) (Buhlin *et al.* 2003, Forner *et al.* 2006). Earlier literature shows that peripheral blood monocytes challenged by bacterial lipopolysacharide produce inflammatory mediators like

IL-6 and TNF-α (Salvi *et al.* 1997, Devaraj *et al.* 2007). Multiple immune cells in the liver, including Kupffer cells, hepatocytes, dendritic cells, and T cells, are known for their capacity to produce cytokines such as IL-6, TNF-α, and IL-10 in response to bacterial LPS (Tiegs & Loshe 2010). In addition to being produced by distant immune cells, one proposed mechanism behind the association between periodontal inflammation and systemic inflammatory burden is "dumping" of locally produced inflammatory mediators into the circulation. Adipose tissue is an important distant source of circulating TNF-α (Ritchie 2007) and IL-6 (Mohamed-Ali *et al.* 1997).

In the light of the above-described biologic pathways, it seems evident that periodontal inflammation increases the systemic inflammatory burden. However, when interpreting the observations of the present IL-6 study (Paper I), a possible bidirectional nature of the association between periodontal and systemic inflammation should be considered. Accordingly, the pro-inflammatory state characterized by increased levels of systemic inflammatory biomarkers may also contribute to increased tissue pathology in the periodontal area (Andriankaja *et al.* 2009). In a respective manner, high systemic levels of anti-inflammatory modulators, such as IL-10 and HDL, could be seen as protective against periodontal inflammation.

6.1.1 Serum IL-6 and periodontal inflammation in T1DM

The positive association between serum IL-6 level and residual periodontal inflammation after therapy on the one hand and the observed lesser reduction of periodontal inflammation in subjects with a higher serum IL-6 level on the other, was interpreted to indicate poorer periodontal healing in subjects with a stronger pro-inflammatory state. Our results are suggestive of a causal pathway, where a high serum IL-6 level acts as a susceptibility factor to periodontal inflammation. The above conclusion is supported by the facts that the association between serum IL-6 level and periodontal inflammation was significant even after adjusting for HbA1c level and that serum IL-6 level was not consistently affected by periodontal therapy. Furthermore, by excluding smoking and overweight subjects (BMI > 26 kg/m^2) we were able to limit many other pathways that increase the risk for periodontal infection. IL-6 is known for its pro-inflammatory properties (Kaplanski *et al.* 2003, Gabay 2006, Kishimoto 2006) and it also accelerates bone resorption (Tamura *et al.* 1993). These biological facts support the proposed conception that a high serum IL-6 level boosts inflammation and modulates tissue

responses locally in the periodontal area as it modulates the development and progression of diabetic micro- and macrovascular complications (Saraheimo *et al.* 2003, Devaraj *et al.* 2007).

Although we consider the above direction of the causal pathway more likely, we cannot fully exclude the opposite pathway, where a high serum IL-6 level is interpreted to reflect a high extent of periodontal inflammation. Supporting this, slightly decreased levels of serum IL-6 (D'Aiuto *et al.* 2005, O'Connell 2008, Kardesler *et al.* 2010) have been reported after anti-infective periodontal therapies. However, in our subjects the decrease in the mean serum IL-6 level from the baseline to the follow-up examination (0.1 pg/ml) was not statistically significant.

6.1.2 Serum TNF-α/IL-10 ratio in subjects with chronic periodontitis

We considered the higher serum TNF-α/IL-10 ratio in the group of subjects with chronic periodontitis when compared with the control subjects to be indicative of a stronger systemic pro-inflammatory state in chronic periodontitis. The ratio between pro- and anti-inflammatory biomarkers, characteristic for each individual, is pivotal also in other inflammatory/infectious conditions, such as myocardial infarction, alcoholic liver disease, or bowel disease, in which a systemic pro-inflammatory state prevails (McClain *et al.* 2004, Karpinski *et al.* 2009, Szkaradkiewicz *et al.* 2009). On the other hand, a low ratio has been considered protective against inflammatory processes. Like IL-6, TNF-α is a pro-inflammatory cytokine that amplifies innate immune responses and fuels tissue pathology towards degradation (Dinarello 1996, Pfizenmaier *et al.* 1996), whereas the immunoregulatory cytokine IL-10 is known to limit and ultimately terminate inflammatory responses, e.g. by suppressing pro-inflammatory cytokine production (de Waal Malefyt 1991, Moore *et al.* 2001, Asadullah *et al.* 2003). Supporting our previous result of serum IL-6 as a modulator of local immune response, it can be speculated that the overall systemic cytokine profile in our periodontitis subjects may have modulated local inflammatory reactions towards increased tissue destruction. In our data a high TNF-α/IL-10 ratio was characteristic of subjects with periodontitis, which is in line with previous studies where down-regulation of IL-10 and up-regulation of TNF-α with increasing extent of periodontal inflammation have been reported (Lin *et al.* 2003, Hyvärinen *et al.* 2009).

Multiple immune cells in the liver are known for their capacity to produce cytokines, for example IL-10 and TNF-α, as a response to LPS from the intestinal

area (McClain *et al.* 2004, Tiegs & Loshe 2010). Whether or not LPS from the periodontal area is able to switch the systemic inflammatory status to the pro-inflammatory side is unclear. As this is a cross-sectional study, no definite conclusions on the direction of the association can be drawn. Moreover, no dose dependency between the extent of periodontal inflammation and TNF-α/IL-10 ratio could be observed—a limitation when the significance of the present results are assessed.

6.1.3 Serum HDL and periodontal inflammation in T1DM

We found an inverse association between serum HDL level and the extent of inflamed periodontal sites in subjects with T1DM and concluded that serum HDL may be considered as a marker for susceptibility to periodontal inflammation. A number of earlier studies have also reported an association between periodontal inflammation and serum lipid profile (Pussinen *et al.* 2004, D'Aiuto *et al.* 2005, D'Aiuto *et al.* 2006, Buhlin *et al.* 2009). Contrary to earlier studies focusing on the effect of periodontal inflammation and/or microbes on the serum lipid profile, we used an opposite analytical strategy with periodontal inflammation as the outcome and serum HDL level as the main explanatory variable. As presented above, the protective role of HDL against inflammation has been shown in earlier studies (Levine *et al.* 1993, Nofer *et al.* 2002). Analogous to coronary heart disease, in which the antioxidant and anti-inflammatory properties of HDL are associated with protection against inflammation (Barter *et al.* 2003, McGrowder *et al.* 2011), our data showed that subjects with a high serum HDL level had fewer periodontally inflamed sites than those with a low level. Based on the categorization of our subjects into HDL tertiles, the protective association of HDL with periodontal inflammation was evident at levels ≥ 1.35 mmol/l. According to current guidelines, the target level of HDL recommended in Finland is ≥ 1.0 mmol/l. Our findings are also in line with previous studies reporting an inverse association between other diabetic complications such as retinopathy (Sasongko *et al.* 2011) or microalbuminuria (Mattock 2001) and decreased plasma HDL or ApoAI, the main constituent of HDL cholesterol.

As this is a cross-sectional study, no definite conclusions on the direction of the studied association can be drawn. Considering the above anti-inflammatory functions of HDL, one could hypothesize that systemic HDL has a protective effect against periodontal inflammation locally. Longitudinal studies to verify a

possible causal relationship between periodontal inflammation and serum HDL level are needed.

6.1.4 GCF MMP-8 and periodontal destruction

MMPs are assumed to primarily play a matrix degrading role, but recent literature suggests that these proteinases can also act as inflammatory mediators and modulate immune responses (McQuibban 2002, Sorsa *et al.* 2004). In addition, MMP-8 can process anti-inflammatory cytokines and chemokines and exert anti-inflammatory effects during host response (Owen 2004). Contrary to its degrading role in periodontitis, MMP-8 has been suggested to play a protective role in lung inflammation by regulating inflammatory cell apoptosis (Gueders *et al.* 2005), cancer progression (Gutierrez-Fernandez *et al.* 2008, Korpi *et al.* 2008), and wound healing (Gutierrez-Fernandez *et al.* 2007).

While previous studies have focused on increased MMP-8 levels in diseased sites (Kinane *et al.* 2003, Uitto *et al.* 2003), we analyzed GCF MMP-8 levels also in healthy sites, and for the first time correlated them with the overall periodontal health status of the subjects. The higher the local MMP-8 level in healthy crevices, the higher the individual extent of periodontal destruction. Therefore, the local MMP-8 level in healthy crevices may be regarded as a marker of how sensitive the inflammatory response is to a microbial challenge. Recent evidence suggests that variation in MMP levels may be due to gene polymorphisms in genes that regulate MMP production, which further on may contribute to disease initiation and progression (Morgan *et al.* 2011, Pradhan-Palikhe *et al.* 2012).

Different antibodies have been used in previous studies of MMP-8 (Kinane *et al.* 2003, Liu *et al.* 2006, Söder *et al.* 2006, Biyikoglu *et al.* 2009). However, we measured the total concentration of MMP-8, including both its active and latent forms, and did not test whether similar results would have been obtained using other MMP-8 antibodies. Moreover, we did not measure the volume of GCF absorbed to the paper strips to assess the quantity of the enzyme present per unit volume of GCF (Lamster *et al.* 1988). Instead, we measured the MMP-8 concentration in ng/ml of the sample (containing the GCF absorbed by the four strips and a total of 200 µl 0.9% saline). While we assume that at the shallow crevices (healthy sites) a fairly constant volume of GCF was absorbed, we were not able to control the influence of various degrees of inflammation on the volume of GCF, which may have caused slight inaccuracy in the reported

concentrations at the diseased sites. Another limitation was that TIMPs were not analyzed.

Due to the cross-sectional study setting, no definite conclusion on a causal relationship can be drawn. However, it cannot be ruled out that the association between the GCF MMP-8 concentration in shallow crevices of chronic periodontitis patients and loss of attachment could be indicative of higher susceptibility to periodontal disease.

6.2 Study design

6.2.1 Subjects

Three different study groups were used in our analyses: 1) T1DM subjects with varying extents of periodontal disease (Papers I, III), 2) subjects with moderate to severe chronic periodontitis (Papers II, IV), and 3) control subjects with clinically healthy periodontal tissues or only minimal signs of gingival inflammation, matched by age and gender with the periodontitis subjects (Paper II).

No a-priori power analyses for study samples were made. The post-hoc power analysis indicated that over all, sufficient numbers of subjects were included. However, due to the use of fairly small sample sizes after stratifications, the preliminary associations now found should be verified using larger samples. Although all the subjects in the chronic periodontitis and control groups were anamnestically healthy, we cannot fully exclude that some of them had subclinical inflammatory/infectious conditions affecting their serum levels of inflammatory markers. In the light of the hyperglycemia-induced systemic pro-inflammatory state and exaggerated immune response of the diabetic subjects, they constitute a special population for studying the associations between local and systemic inflammation. We considered that any significant associations between periodontal inflammation and systemic inflammatory markers observed in a complicated situation like diabetes mellitus would be indicative of the strength of these associations. As no non-diabetic group with similar periodontal health status was included, it was not possible to assess the influence of T1DM on the studied associations.

6.2.2 Methods

Both longitudinal (Paper I) and cross-sectional (Papers II-IV) study designs were used. In the longitudinal study, the diabetic subjects were periodontally treated after a baseline examination and a clinical re-examination was performed eight weeks after completion of the treatment, allowing us to draw conclusions on the possible bidirectional nature of the association between periodontal health and systemic inflammatory status. In three studies (Papers II-IV), a cross-sectional study design was used, prohibiting us from drawing definite conclusions on the direction of the observed associations between periodontal health and the inflammatory markers studied (IL-10, TNF-α, HDL, MMP-8).

The laboratory methods included both commercially available ELISA assays (IL-6, IL-10, TNF-α, and MMP-8) and routine laboratory techniques (HDL, LDL, triglycerides, HbA1c, and usCRP). The skewness of the IL-10, TNF-α, and HDL data was checked before the analyses. Due to the skewed distribution of the TNF-α and IL-10 level data, a logarithmic transformation was performed to yield a symmetrical distribution.

All the subjects were examined by an experienced periodontal specialist following detailed calibration of the clinical examination procedure. Measurements of BOP and pocket depth (PD ≥ 4 mm, PD ≥ 6 mm), either separately or in combination with each other (bleeding and PD ≥ 4 mm), were used to describe current periodontal inflammation. To indicate a long-term predisposition to periodontal disease, attachment level measurements (AL ≥ 4 mm, AL ≥ 6 mm) were used. The uniformity of the results using various periodontal variables can be seen as indicative of the validity of the results. BOP was normally distributed, whereas pocket depth and attachment level data were slightly skewed, and both negative binomial and linear regression models were used with essentially the same results.

The outcome variables were primarily used as continuous variables. To get a better idea of the linearity of the associations, explanatory variables were used as both continuous and categorized. As no generally accepted cut-off points for various categories were available, categorization into tertiles was used.

6.2.3 Confounding factors

Confounding and modifying factors were considered in analyzing the strengths of the various associations. Selection of the confounding factors was based on

unadjusted correlations. We focused particularly on the factors associated with both the outcome and the main explanatory variables. In addition, we studied whether the confounding factors, such as smoking or BMI, had any modifying effects on the studied associations. Possible modifying effects were studied in the first phase by using interaction terms in the regression models, and subsequent stratifications were made. Due to the small sample size, simultaneous stratification by multiple factors, such as smoking and BMI, was not possible. This had hardly any significant effect on our main results.

Smoking is a well-established, independent risk factor for periodontal tissue destruction associated strongly with clinical periodontal parameters (Bergström 2006, Johnson & Guthmiller 2007). In addition, cigarette smoking seems to alter the composition of bacterial dental plaque in a more pathogenic direction (Shiloah *et al.* 2000). Substantial evidence suggests that smoking impairs various aspects of innate and adaptive immune responses, such as neutrophil function, antibody production, fibroblast activities, and production of inflammatory mediators (Johnson & Guthmiller 2007). Controlling for smoking using a dichotomous variable (smoker/non-smoker) is questionable because it is well known that this type of variable may leave significant residual confounding in the associations studied. A better way to control for the effect of smoking is to exclude smokers or at least use more definite measures of smoking, such as pack-years. We used stratification whenever possible. The results based on regression models using a dichotomized smoking variable should be verified in future studies.

Several cross-sectional studies have demonstrated an association between periodontitis and obesity (Ylöstalo *et al.* 2008, Han *et al.* 2010, Saxlin *et al.* 2011). As obesity has been found to induce low-grade systemic inflammation (Ritchie 2007) and adipocytes are known to produce many cytokines, especially IL-6 and TNF-α (Falagas & Kompoti 2006), it was pivotal to control for BMI. Controlling for obesity using BMI as a continuous variable in the models was considered sufficient. In addition to using BMI as a continuous variable in the regression model, stratification according to BMI was used in the IL-6 study.

In the analyses concerning associations between periodontal inflammation and systemic inflammatory status in the T1DM subjects, adjustments were also made with regard to the level of HbA1c, a well-known measure of diabetic status on one hand and a significant determinant of both gingivitis and periodontitis on the other hand (Taylor 2001, Taylor & Borgnakke 2008). Moreover, diabetic status is known to be associated with systemic inflammation (Devaraj *et al.* 2007, Kaul *et al.* 2010).

7 Conclusions

Serum levels of inflammatory markers/mediators reflect the degree of systemic inflammatory burden, and increased levels have been found in many inflammatory diseases such as cardiovascular diseases and rheumatoid arthritis.

Unlike most previous studies where periodontal infection has been shown to propagate low-grade systemic inflammation, this study together with the findings of Adriankaja and his group (Andriankaja *et al.* 2009) unambiguously indicates that serum IL-6 has a modulatory role in local host responses in the periodontal area. Therefore, serum IL-6 could be a candidate marker in determining a subject's susceptibility to gingivitis and periodontitis as well as in prognosis of periodontal therapy.

The mean TNF-α/IL-10 ratio of 0.6 (95% CI 0.4-0.8) observed in periodontally healthy control subjects seems to present a balance between tissue regeneration and degradation in periodontal tissue. An approximately three times higher ratio was characteristic of subjects with periodontitis. The clinical relevance of these interesting and preliminary findings needs to be confirmed in a longitudinal study setting, for example using periodontal treatment intervention. Such a study would also clarify whether serum TNF-α/IL-10 ratio, similar to serum IL-6 level, could also reflect a subject's susceptibility to gingivitis and periodontitis.

Subjects with a serum HDL level < 1.35 mmol/l presented 50% more sites with bleeding and PD ≥ 4 mm when compared with subjects with serum HDL $\geq$ 1.35 mmol/l. According to current guidelines in Finland, the target level of HDL is ≥ 1.0 mmol/l. Further longitudinal studies of non-diabetic subjects are needed to verify the possible causality of the association, and to study other possible protective properties of HDL against periodontal inflammation.

Earlier studies have focused on the role of MMP-8 in periodontal destruction, but according to our results, GCF MMP-8 in shallow crevices could possibly be used as a marker of existing periodontal destruction. A longitudinal study would be needed to verify whether this marker has prognostic value.

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List of original articles

This thesis is based on the following original publications, which are referred to in this thesis by Roman numerals:

I Passoja A, Knuuttila M, Hiltunen L, Karttunen R, Niemelä O, Raunio T, Vainio O, Hedberg P & Tervonen T (2011) Serum interleukin-6 may modulate periodontal inflammation in type 1 diabetic subjects. J Clin Periodontol 38: 687–693.

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