

Taina Raunio

GENE POLYMORPHISM AND
SYSTEMIC INFLAMMATORY
RESPONSE IN CHRONIC
PERIODONTITIS

FACULTY OF MEDICINE,
INSTITUTE OF DENTISTRY, DEPARTMENT OF PERIODONTOLOGY AND GERIATRIC DENTISTRY,
INSTITUTE OF DIAGNOSTICS, DEPARTMENT OF MEDICAL MICROBIOLOGY,
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TAINA RAUNIO

**GENE POLYMORPHISM AND
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PERIODONTITIS**

Academic dissertation to be presented with the assent of the Faculty of Medicine of the University of Oulu for public defence in Auditorium 1 of the Institute of Dentistry (Aapistie 3), on 30 October 2009, at 12 noon

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Supervised by
Professor Matti Knuutila
Docent Tellervo Tervonen
Docent Riitta Karttunen

Reviewed by
Professor Mikko Hurme
Professor Veli-Jukka Uitto

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Faculty of Medicine, Institute of Dentistry, Department of Periodontology and Geriatric Dentistry, University of Oulu, P. O. Box 5281, FI-90014 University of Oulu, Finland, Institute of Diagnostics, Department of Medical Microbiology, University of Oulu, P.O.Box 5000, FI-90014 University of Oulu, Finland

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Abstract

In this study, associations between periodontitis expression, serum levels of inflammatory markers and genetic factors were investigated. The periodontal status of 56 subjects with chronic periodontitis, 28 control subjects and 80 subjects with type I diabetes mellitus (DM) was examined. In addition, a reference group (n=178) with genetic but not with periodontal health data was included. The single nucleotide polymorphisms of *CD14* -260, *IL-6* -174, *TNF- α* -308, *IL-10* -1082, *IL-1A* -889, *IL-1B* +3954, and *TLR4* +896 were determined using PCR with RFLP or allele-specific primers, and comparisons of the genotype frequencies were made between the study groups and reference subjects. The serum concentrations of IL-6 and sCD14 were assayed using ELISA.

The distributions of all the studied genotypes were similar in the periodontitis and the reference subjects. However, in the periodontitis group, the carriage of the T-containing genotype of the *CD14* -260 and the GG genotype of the *IL-6* -174 associated significantly with the extent of periodontitis, indicating that genetic factors play a role in the pathogenesis of the disease.

Both the extent of periodontal infection and the *IL-6* -174 genotype were significant determinants for the serum IL-6 level, subjects carrying the GG genotype having significantly higher serum IL-6 levels than those carrying the CC/CG genotype. The serum level of sCD14 was significantly higher in subjects carrying the T-containing than the CC genotype of the *CD14* -260 in the control group but not in the periodontitis group, suggesting that severe periodontal infection overshadows the influence of the genotype on serum sCD14 level. Overall, the serum studies indicated that periodontal infection is associated with a low-grade systemic inflammatory response.

Type 1 DM subjects carrying the GG genotype of the *IL-6* -174 had a higher extent of periodontitis when compared with those carrying the CG/CC genotype. Our results also suggest that the *IL-6* -174 genotype is a more significant determinant of the extent of periodontitis in type 1 DM than glycemic control.

Keywords: cell surface receptors, cytokines, periodontal disease/blood, periodontal disease/genetics

To my children

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Abbreviations

AGE	advanced glycated end product
AL	attachment level
BL	bone level
BMI	body mass index
BOP	bleeding on probing
CD	cluster of differentiation
CRP	C-reactive protein
DM	diabetes mellitus
HbA1c	hemoglobin A1c
hsCRP	high-sensitivity C-reactive protein
IFN	interferon
IL	interleukin
IL1-Ra	interleukin 1 receptor antagonist
LD	linkage disequilibrium
LPS	lipopolysaccharide
PAMP	pathogen-associated molecular pattern
PD	pocket depth
RAGE	receptor for advanced glycated end product
sCD14	soluble CD14
SNP	single nucleotide polymorphism
TGF	transforming growth factor
Th	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor

List of original articles

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

- I Tervonen T, Raunio T, Knuutila M & Karttunen R (2007) Polymorphisms in the CD14 and IL-6 genes associated with periodontal disease. *J Clin Periodontol* 34: 377–383.
- II Raunio T, Nixdorf M, Knuutila M, Karttunen R, Vainio O & Tervonen T (2007) The extent of periodontal disease and IL-6⁻¹⁷⁴ genotype as determinants of serum IL-6 level. *J Clin Periodontol* 34: 1025–1030.
- III Raunio T, Karttunen R, Knuutila M, Vainio O & Tervonen T (2009) Serum sCD14, polymorphism of CD14⁻²⁶⁰ and periodontal infection. *Oral Dis* 15: 484–489.
- IV Raunio T, Knuutila M, Hiltunen L, Karttunen R, Vainio O & Tervonen T (2009) The IL6⁻¹⁷⁴ genotype associated with the extent of periodontal disease in type 1 diabetic subjects. *J Clin Periodontol* 36: 11–17.

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1 Introduction

According to the Health 2000 Survey conducted in 2000–2001 in Finland, periodontitis was found to be the most common oral disease among the adult population. Of all dentate subjects aged 30 years and over, 64% had periodontitis, the prevalence of severe forms being 21% (Suominen-Taipale *et al.* 2008). Overall, in Finland the prevalence figures of periodontitis are slightly higher than in most Western countries (Albandar 2002, Sheiham & Netuveli 2002).

Bacteria are essential in the etiology of periodontal infection, but subsequent progression and disease severity are thought to be determined by the immune responses of the host, environmental modifiers and genetic factors, such as gene polymorphisms. There is growing evidence that genes influence an individual's susceptibility to the initiation and progression of periodontal disease. As a multifactorial disease, periodontitis has typically multiple gene associations, which individually have weak effects but when combined with other influences, such as environmental factors, result in variable disease manifestation (Takashiba & Naruishi 2006). Different allelic variants can result in variations e.g. in the amounts of inflammatory mediators and may act as protective or risk factors for the disease (Yoshie *et al.* 2007).

In the past decade, a vast number of studies have focused on evaluating the relationship between periodontal disease and systemic conditions, such as cardiovascular diseases and diabetes mellitus. In addition to genetic traits, a chronic systemic low-grade inflammation has been suggested to be a common link behind these conditions. Obviously, bacteria and bacterial toxins have access to the blood stream in periodontitis (Forner *et al.* 2006a). As a consequence of this bacterial irritation and also of the systemic release of inflammatory mediators from the periodontal area, there is an increase in the levels of inflammatory mediators in peripheral blood (Loos 2005).

In the background of the present study are the previously known positive associations between the presence of local or systemic inflammatory diseases and serum levels of inflammatory mediators as well as obvious regulatory effects of genetic factors on these associations. In the context of this study the associations between the severity of chronic periodontitis, genetic polymorphism and serum levels of inflammatory mediators were studied.

2 Review of the literature

2.1 Host responses in periodontal disease

Periodontitis is a common, chronic, and complex inflammatory disease caused by bacterial biofilms that accumulate on the tooth surface and gingival sulcus, and it is characterized by progressive destruction of the structures that support teeth (Kornman 2008, Van Dyke 2007). Although periodontal diseases are initiated by bacteria, the host response is believed to play an essential role in the breakdown of connective tissue and bone. Microbial antigens and virulence factors elicit an inflammatory and immune reaction, in which both innate and adaptive immune responses are involved (Van Dyke & Kornman 2008, Graves 2008). The response varies among individuals, depending on potential variations in cytokine and other antimicrobial responses, environmental factors, and the subjects' genetics (Kinane *et al.* 2007, Gemmell *et al.* 2007).

In healthy subjects, the epithelial barrier in the oral cavity together with the protective innate immune molecules inhibits oral bacteria from entering into the tissues and the bloodstream (Moutsopoulos & Madianos 2006). In this respect periodontal disease may in most cases be considered a localized infection. However, it is speculated that the inflamed and ulcerated subgingival pocket epithelium could form an easy port of entry for dental plaque bacteria to disperse systemically (Moutsopoulos & Madianos 2006, Hujoel *et al.* 2001). Indeed, many systemic markers of inflammation in periodontitis have been reported, and this condition has also been suggested to be linked to systemic diseases (Loos 2005). The described associations have been explained by bacterial seeding, common inflammatory mechanisms and modifying factors (Kornman 2008). Systemic diseases, such as diabetes mellitus, also modify the host response and may be important determinants of the variation in susceptibility to periodontitis (Nassar *et al.* 2007).

2.1.1 Innate and adaptive immunity in periodontal infection

The first line of defense in infectious diseases, the innate immunity, is challenged to detect pathogens and mount a rapid defensive response, which requires no prior immune learning or experience. It is responsible for the defense during the initial hours and days of the infection (Kinane *et al.* 2007, Kirkwood *et al.* 2006). It acts

through the recruitment of immune cells, activation of complement system, identification and removal of foreign substances, and activation of the adaptive immune system (Van Dyke & Kornman 2008).

Neutrophils (PMNs, polymorphonuclear neutrophilic leukocytes) are the first cells to encounter pathogens, soon reinforced by the recruitment of monocytes/macrophages (Janeway *et al.* 2005). These cells recognize pathogens by means of a limited number of cell-surface receptors, such as the macrophage mannose receptors, scavenger receptors and CD14. Ligation of these receptors leads to phagocytosis of the pathogen and secretion of cytokines, such as interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-12 (IL-12) and tumor necrosis factor- α (TNF- α), which are biologically active cell proteins that affect the behavior of other cells that bear receptors for them. Macrophages and other cells, such as lymphocytes, osteoclasts and epithelial cells, release chemokines, such as interleukin-8 (IL-8), that attract more neutrophils and monocytes to the site of inflammation (Graves 2008, Janeway *et al.* 2005, Janeway & Medzhitov 2002). In addition to the cellular innate immune response, plasma proteins accumulate at the site of infection including the complement components that provide circulating or humoral innate immunity. The complement system interacts with microorganisms to promote their removal by phagocytic cells, and it is also considered to constitute a link between the innate and adaptive immune responses (Gasque 2004).

The innate immunity system is closely linked to the adaptive immune response, which helps clear the infection and builds specific immunity with a memory component. Activation of the adaptive response occurs through cytokine secretion, antigenic processing and presentation and differentiation of effector cells (Hornet *et al.* 2002). The primary cells involved in the adaptive immune response are lymphocytes, B cells and T cells. B cells are mainly antibody producers, while T cells are functionally divided into two main classes. One class differentiates on activation into cytotoxic T cells, which kill cells infected with viruses and express CD8 molecule on their cell surfaces. T helper cells, on the other hand, are marked by the expression of the cell-surface molecule CD4, and play an important role in the initiation of immune responses by providing help to other cells (Janeway *et al.* 2005). Upon antigenic stimulation, naive CD4⁺ T cells proliferate and differentiate into different effector cells, T helper type 1 (Th1) and type 2 (Th2) cells, characterized by the production of specific cytokines and effector functions (Korn *et al.* 2007, Mosmann & Coffman 1989). Th1 cells produce large quantities of interferon (IFN)- γ , elicit delayed-type hypersensitivity

responses, activate macrophages and CD8⁺ cells, and effectively clear intracellular pathogens. Other cytokines, such as IL-12, IL-2, lymphotoxin (TNF- β), and IL-10 have also been called Th1 cytokines. Th2 cells produce interleukin 4 (IL-4), IL-5, IL-13 and IL-25, mediate humoral immunity including production of IgE, and activate eosinophilic inflammation and the clearance of helminth parasitic infections (Korn *et al.* 2007, Gaffen & Hajishengallis 2008).

The Th1/Th2 dichotomy has recently been revised. As with many other inflammatory diseases, the observed role of T-cell mediated immunity in periodontitis has not entirely fitted the classic Th1/Th2 model (Gaffen & Hajishengallis 2008). A new subset of CD4⁺ T-cells has been discovered, and it explains discrepancies in the classic Th1/Th2 model. It is termed Th17 based on its secretion of the proinflammatory cytokine IL-17. Activators of Th 17 differentiation are transforming growth factor- β (TGF- β), IL-1, IL-6 and IL-21, whereas IL-23 is required for Th17 cell expansion, survival, and pathogenicity (Gaffen & Hajishengallis 2008, Stockinger & Veldhoen 2007).

T cells are considered to have a central role in controlling the progression of periodontal disease, and different types of T cell clones have been demonstrated to play destructive roles (Taubman & Kawai 2001). The early periodontal lesion (gingivitis) is characterized by increased numbers of T cells, but there is only limited consensus regarding the nature of Th cells that predominate in inflamed gingival tissue. Most of the studies support the notion that Th1 cells and their cytokines are associated with early/stable periodontal lesions, while Th2-type response in periodontium predisposes to susceptibility to disease progression (Gemmell *et al.* 2007). There is also increasing evidence that Th17 cytokines participate in periodontal disease, but whether their primary role is host-protective or destructive is not clear (Gaffen & Hajishengallis 2008).

Although T cells act as the main regulatory group of cells in periodontitis lesions, B cells have several critical roles. After encounter with antigen, B cells differentiate into antibody-secreting plasma cells. The production of antibodies involves the response to foreign antigens as well as to self antigens (Berglundh *et al.* 2007). In periodontitis lesions plasma cells represent about 50% of all inflammatory cells, while B cells comprise about 18%. The proportion of B cells is larger than that of all T cells, and CD4⁺ T cells occur in larger numbers than CD8⁺ T cells (Berglundh & Donati 2005). Plasma cells also produce cytokines such as tumor necrosis factor- α , interleukin-6, interleukin-10 and transforming growth factor- β (Berglundh *et al.* 2007).

2.1.2 Cytokines and receptor molecules involved in periodontal inflammation

Cytokines are biologically active molecules released by specific cells that elicit a particular response from other cells on which they act. The responses caused by these substances are varied and interrelated. In general, cytokines control growth, mobility and differentiation of lymphocytes, but they also exert a similar effect on other leukocytes and some non-immune cells (Greenwold *et al.* 2007).

Cytokines are effective in very low concentrations, are produced transiently, act locally in the tissue where they are produced (Page *et al.* 1997), and can also have systemic effects (Okada & Murakami 1998). They are often self-regulatory and able to induce their own expression in an autocrine (affecting the behavior of the cell that releases the cytokine) or paracrine fashion (affecting the behavior of adjacent or even distant cells). Cytokines act on their target cells by binding to specific receptors and initiating intracellular second messengers resulting in phenotypic changes in the cell via altered gene regulation (Taylor *et al.* 2004). There is a significant overlap and redundancy between the function of individual cytokines. They do not act in isolation, but rather as a complex network, bringing together elements of both innate and adaptive immunity (Banyer *et al.* 2000).

Cytokines play an important role in a number of different physiologic processes, but if expressed inappropriately, they also induce pathology. An inflammatory cytokine is defined as a cytokine which is induced during the course of an inflammatory response and is closely associated with its onset and/or progression. IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α are generally classified as pro-inflammatory cytokines (Okada & Murakami 1998). Pro-inflammatory cytokines enhance the bactericidal capacity of phagocytes, recruit additional innate cell populations to sites of infection, induce dendritic cell maturation and direct the ensuing specific immune response to the invading microbes (Hornef *et al.* 2002). Anti-inflammatory cytokines block this process or at least suppress the intensity of the cascade. Cytokines such as IL-4, IL-10, IL-13 and transforming growth factor (TGF) - β suppress the production of IL-1, TNF, chemokines such as IL-8, and vascular adhesion molecules (Dinarello 2000).

Under pathologic conditions such as those that occur in periodontal disease, the balance between pro- and anti-inflammation is directed towards proinflammatory activity (Graves & Cochran 2003). In periodontal tissue destruction three proinflammatory cytokines, interleukin-1 (IL-1), IL-6, and

tumor necrosis factor- α (TNF- α), appear to have a central role (Nikolopoulos *et al.* 2008).

The three cytokines originally described as the members of the IL-1 family are IL-1 α and IL-1 β , which have agonist activity, and IL-1Ra, a physiologic antagonist to other IL-1 cytokines. They are secreted by a variety of cell types including monocytes, macrophages, dendritic cells, epithelial cells, keratinocytes and fibroblasts. IL-1 α is a regulator of intracellular events and a mediator of local inflammation, whereas IL-1 β is primarily an extracellular protein released from cells (Barksby *et al.* 2007, Dinarello 1997).

In periodontal tissues IL-1 is known to stimulate the proliferation of keratinocytes, fibroblasts, endothelial cells and to enhance fibroblast synthesis of type I procollagen, collagenase, hyaluronate, fibronectin, and prostaglandin E₂. IL-1 is therefore a critical component in the homeostasis of periodontal tissues, and its unrestricted production may lead to tissue damage (Okada & Murakami 1998). It has been demonstrated that a significant correlation exists between the IL-1 β levels of gingival crevicular fluid (GCF) and periodontal parameters such as probing pocket depth and attachment level (Orozco *et al.* 2006, Engebretson *et al.* 2002, Figueredo *et al.* 1999, Masada *et al.* 1990). IL-1 β upregulates matrix metalloproteinases and downregulates tissue inhibitors of metalloproteinase production (Page *et al.* 1997), and it is also a potent stimulator of bone resorption (Shirodaria *et al.* 2000).

IL-6 was originally identified as a B-cell stimulatory and differentiation factor, but is currently known as a regulator of the immune response, hematopoiesis, acute phase response and inflammation (Kishimoto 2006, Gabay 2006). IL-6 is produced by a number of cells including monocytes/macrophages, activated T-cells, endothelial cells, adipocytes and fibroblasts (Kishimoto 2006, Gabay 2006). One of the most important systemic actions of IL-6 is induction of acute-phase proteins such as CRP and serum amyloid A, which are primarily produced by the liver and promote the immune response through activation of complement, induction of proinflammatory cytokines, and stimulation of neutrophil chemotaxis (Gabay 2006, Cronstein 2007). IL-6 synthesis and release are stimulated by two major proinflammatory cytokines, IL-1 β and TNF- α , but the fact that IL-6 remains substantially longer in the plasma makes this molecule a good marker of inflammation (Song & Kellum 2005). IL-6 also plays a role in the transition between acute and chronic inflammation. A recently described effector T-cell subset, Th17, will only differentiate from naive T cells in the presence of IL-6 and TGF- β (Stockinger & Veldhoen 2007). Other biological

activities of IL-6 include the enhancement of T-cell proliferation and acceleration of bone resorption by increasing osteoclast formation (Ishimi *et al.* 1990, Tamura *et al.* 1993). IL-6 has also other biological non-immunological effects, such as the regulation of tumorigenesis by enhancing proliferation of tumor-initiating cells and protecting cells from apoptosis (Pucci *et al.* 2009, Grivennikov *et al.* 2009), contribution to insulin metabolism (Yuen *et al.* 2009) or the secretion of other hormones (Renner *et al.* 2009).

IL-6 is produced locally in the inflamed tissues following cellular activation by bacterial lipopolysaccharide (LPS) or other cytokines such as IL-1 β or TNF- α (Kishimoto 1989, Ishihara & Hirano 2002). Local production of IL-6 also occurs in inflamed periodontal tissues (Takahashi *et al.* 1994, Dongari-Bagtzoglou & Ebersole 1998) and significant correlations have been found between periodontal pocket depth and IL-6 -content in gingival crevicular fluid (Geivelis *et al.* 1993, McGee *et al.* 1998). IL-6 has also been associated with attachment loss in refractory periodontitis (Reinhardt *et al.* 1993) and found in elevated levels in gingival connective tissue adjacent to intrabony pockets representing poor response to non-surgical periodontal treatment (Guillot *et al.* 1995).

TNF is found in two forms, TNF- α and TNF- β (Graves & Cochran 2003), and the synergism of IL-1 and TNF is a commonly reported phenomenon (Dinarello 2000). IL-1 and TNF are produced by the same cell types (Graves & Cochran 2003), they often affect together, and many of their pathways are shared (Dinarello 2000), including inflammatory bone resorption (Gravallese *et al.* 2001). In the development of Th17 response TNF- α and IL-1 β are found to amplify the response induced by TGF- β and IL-6, but are not able to substitute for either of these cytokines (Stockinger & Veldhoen 2007, Weaver *et al.* 2006). Furthermore, IL-1 and TNF can induce upregulation of adhesion molecules on leukocytes and endothelial cells and stimulate the production of chemokines and other inflammatory mediators, such as prostaglandins, and lytic enzymes, such as matrix metalloproteinases (Graves & Cochran 2003, Dinarello 1997) and their inhibitors (Berglundh *et al.* 2007). Cytokines may also reduce the capacity to repair the damaged periodontal tissue through apoptosis of resident cells, such as fibroblasts (Graves *et al.* 2001).

In a complex network of pro- and anti-inflammatory cytokines acting in the inflamed periodontal tissues, interleukin-10 is an example of a cytokine with anti-inflammatory effects. Interleukin-10 is a regulatory cytokine, which on the one hand limits inflammatory responses by inhibiting the expression of pro-inflammatory cytokines (e.g. IL-1 α , IL-1 β , IL-6, TNF- α), but also upregulates the

recruitment and activation of B cells (Moore *et al.* 2001). It downregulates the T helper 1 response, and by controlling the B-cell lesion (Moore *et al.* 2001) it is suggested to play a role in controlling the progression of periodontal disease (Cullinan *et al.* 2008). In a study where several key cytokines were assessed from inflamed gingival biopsies, high absolute levels of IL-1 β , TNF- α , IL-2 and IFN- γ , and especially high ratios of IL-1 β and IL-10, as well as TNF- α and IL-4, were found in tissue biopsies from periodontitis patients, and they correlated strongly with the severity of periodontal disease (Gorska *et al.* 2003).

The innate immune system recognizes common pathogen-associated molecular patterns (PAMPs) that are expressed on micro-organisms, but not on host cells, by extra- and intracellular receptors like CD14, Card15 and Toll-like receptors (TLRs). They identify PAMPs of Gram-positive and Gram-negative bacteria and mediate the production of cytokines necessary for further development of effective immunity (Dixon *et al.* 2004, Loos *et al.* 2005).

Toll-like receptor 2 and Toll-like receptor 4 are two principal pattern-recognition receptors that detect bacterial cell wall components, such as lipoproteins and lipopolysaccharides (LPS). CD14 is an important co-receptor, which acts on the cell membrane by facilitating interaction between LPS, lipoteichoic acids (LTA) or other bacterial ligands and Toll-like receptors. CD14 principally acts to bind LPS and transfers it from circulating LPS-binding protein to the Toll-like receptor 4/MD-2 signaling complex (Dixon *et al.* 2004, Kitchens & Thompson 2005, da Silva Correia *et al.* 2001, Medzhitov & Janeway 2000). Signal transduction includes recruitment of adaptor protein MyD88 and IL-1 receptor associated kinase (IRAK) and results in nuclear translocation and gene activation of proinflammatory cytokines (da Silva Correia *et al.* 2001, Arroyo-Espliguero *et al.* 2004, Anderson 2000).

The CD14 protein is found in two distinct forms: membrane CD14 (mCD14), expressed primarily on the surface of monocytes/macrophages and neutrophils, and a soluble form (sCD14) that lacks the glycosylphosphatidylinositol anchor (Bas *et al.* 2004, Le Van *et al.* 2001, Bazil *et al.* 1986). Soluble CD14 mediates the response to lipopolysaccharide (LPS) in cells lacking membrane-bound CD 14, such as endothelial and epithelial cells (Le Van *et al.* 2001).

Besides its function mainly in enhancing inflammatory responses, sCD14 may also act as a downregulatory molecule by controlling the immune system level of response in inflammatory diseases (Kitchens & Thompson 2005, Bas *et al.* 2004). sCD14 inhibits cell responses by diverting LPS from mCD14 and transferring it to plasma lipoproteins, and even normal sCD14 concentrations

have that suppressive effect (Kitchens & Thompson 2005). It has also been demonstrated that sCD14 is a regulatory factor capable of modulating cellular and humoral immune responses by interacting directly with T and B cells (Arias *et al.* 2000).

In periodontitis patients, local production of sCD14 may contribute to its levels in gingival crevicular fluid and saliva. In a study where sCD14 levels were determined in gingival crevicular fluid from 15 subjects, sCD14 expression was found to be negatively correlated with the amount of probing depth, thus suggesting a local protective effect for sCD14 (Jin & Darveau 2001). On the contrary, in a recent report a statistically significant correlation was found between salivary sCD14 concentration and clinical periodontal parameters, including the extent of periodontal disease. Concentration of sCD14 in parotid saliva was reported to be comparable to that in serum, and inflammatory mediators were suggested to be washed into saliva from gingival crevicular fluid (Isaza-Guzman *et al.* 2008).

2.2 Systemic response to periodontal infection

According to knowledge acquired recently, a low-grade inflammation is a potential risk factor for several systemic conditions including cardiovascular disease, diabetes mellitus and pregnancy complications. Local chronic infections, such as periodontal disease, may be seen as potential contributors to the pathogenesis of these systemic diseases (Loos 2005, Moutsopoulos & Madianos 2006).

In periodontitis, the presence of subgingival pathogens first elicits a local inflammatory response, which is characterized by the formation of a local inflammatory infiltrate and migration of a large number of leukocytes towards the affected area. The inflammatory response is then amplified due to the production of pro-inflammatory cytokines and prostaglandins. Bacterial components, such as major outer membrane proteins and endotoxins (i.e. lipopolysaccharide, LPS) may be dispersed into circulation and are eventually suggested to trigger a systemic host response (Loos 2005, D'Aiuto *et al.* 2005, Nibali *et al.* 2007a). Surface material independent of outer membrane vesicles has also been demonstrated to be released from live bacterial cells and to induce proinflammatory response in human whole blood (Oscarsson *et al.* 2008). In response to bacteremia and bacterial antigens, red and white blood cells as well as tissue cells at locations where the antigens are relocated, such as endothelial cells

and hepatocytes, may produce proinflammatory immune mediators (Loos 2005). Furthermore, cytokines such as IL-1, IL-6 and TNF- α produced at the gingival sites may be transported into the systemic circulation and stimulate hepatocytes in the liver to produce increasing levels of acute phase reactants such as C-reactive protein (CRP) (Gabay 2006, Nibali *et al.* 2007a, Ide *et al.* 2004).

The total surface area of all periodontal lesions in an untreated patient with severe periodontitis may amount to up to 20 cm²; a range between 1 and 44 cm² has been reported, and the ulcerated subgingival pocket epithelium allows bacterial cells or their products to enter the systemic circulation (Loos 2005, Hujuel *et al.* 2001). Bacteremia in periodontitis has been reported after oral examination (Daly *et al.* 2001), chewing (Forner *et al.* 2006a, Geerts *et al.* 2002), tooth brushing or scaling (Forner *et al.* 2006a), and signs of viable periodontal pathogens have been demonstrated in human atherosclerotic plaque material (Kozarov *et al.* 2005).

The systemic inflammatory response associated with periodontal disease can be measured as modest changes in the levels of serum markers such as concentrations of acute phase proteins or cytokines (Loos 2005). The best-known acute-phase reactant is C-reactive protein (CRP), which measured as high-sensitivity CRP (hsCRP), provides a biomarker for low-grade systemic inflammation (Ridker & Silvertown 2008, Paraskevas *et al.* 2008). High-sensitivity assays for CRP determine levels in plasma as low as 0.3 mg/L, and in chronic infections such as chronic stomach ulcers associated with *Helicobacter pylori*, CRP has been found at relatively low levels (range 0.3 to 3.0 mg/L) (Loos 2005). The recent systematic meta-analyses on CRP in relation to periodontitis revealed that there is strong evidence from cross-sectional studies that plasma CRP in periodontitis is elevated compared with controls. The weighted mean difference between patients and controls was 1.56 mg/L ($p < 0.00001$). Periodontal treatment has been reported to decrease hs-CRP, but the overall evidence for that is modest (Paraskevas *et al.* 2008).

Cytokines are present in plasma in extremely low concentrations. If present at high levels, they would have profound effects, such as fever and severe overall illness, which are not symptoms of chronic infections (Loos 2005). IL-6 serum levels are found to independently predict future coronary events, although correlations are not as strong as with CRP (Ridker *et al.* 2000b, Ridker *et al.* 2000a). Subjects with no known medical disorders other than periodontitis have been reported to have elevated serum levels of IL-6 when compared to periodontally healthy controls (Buhlin *et al.* 2003, Loos *et al.* 2000). Immediately

after subgingival instrumentation with hand instruments or either sonic or ultrasonic scalers serum IL-6 is known to increase (Ide *et al.* 2004, D'Aiuto *et al.* 2004a, Forner *et al.* 2006b), and resolution of periodontal inflammation has been reported to result in significant decreases in serum IL-6 levels six months after periodontal therapy (D'Aiuto *et al.* 2004b).

A transient increase in circulating tumor necrosis factor- α has been reported following scaling and root planning (Ide *et al.* 2004), and circulating TNF- α has also been reported to associate with the severity of periodontal breakdown (Ide *et al.* 2004, Bretz *et al.* 2005). In type 2 diabetes patients elevated TNF- α levels have been suggested to contribute to insulin resistance (Mishima *et al.* 2001). The source of plasma TNF has been thought to be adipocytes associated with obesity, but inflammation and infection may result in TNF-production as well (Engebretson *et al.* 2007). In a study with type 2 diabetes patients a dose-response relationship was observed between periodontitis severity and circulating TNF- α , and attachment loss and plasma endotoxin were reported to be significant independent predictors for elevated TNF plasma levels (Engebretson *et al.* 2007).

There are only a few reports of the plasma levels of other cytokines (e.g.IL-1 TNF- α , and IL-4) analyzed in periodontitis patients, and mostly the cytokine levels did not differ between cases and controls or correlate with periodontal parameters (Gorska *et al.* 2003, Mengel *et al.* 2002).

Soluble CD14 (sCD14) is shed from monocytes and secreted by hepatocytes, and it is found in normal serum at microgram levels (Bazil *et al.* 1986). The concentration of sCD14 in serum is elevated in many systemic inflammatory, immunological and infectious diseases (Landmann *et al.* 1995, Yu *et al.* 1998, Nockher *et al.* 1999, Garty *et al.* 2000, Martin *et al.* 2006), and sCD14 levels in plasma are known to rise rapidly during endotoxemia (Landmann *et al.* 1995). sCD14 can thus be considered to be an acute-phase protein, and its levels correlate with those of C-reactive protein and IL-6 (Bas *et al.* 2004).

Patients with periodontitis have been reported to have increased serum concentrations of sCD14 compared with periodontally healthy control subjects, and sCD14 levels were shown to decrease after active periodontal treatment (Hayashi *et al.* 1999). Contradictory to the above, no statistically significant differences were found in the mean serum sCD14 concentration between patients with generalized aggressive periodontitis and periodontally healthy control subjects (Pietruska *et al.* 2006).

2.3 Genetic variation in the immune response towards periodontal infection

2.3.1 Gene polymorphism in complex diseases

Most human diseases have a genetic component in their etiology. In that context diseases are genetically divided into simple Mendelian type or complex diseases. Mendelian diseases occur in simple patterns in families, and in most cases a single gene locus is the major determinant of the clinical disease phenotype (Burgner *et al.* 2006, Kinane & Hart 2003, Tabor *et al.* 2002).

A feature that complex human diseases, such as cardiovascular diseases, osteoporosis, Alzheimer's disease and periodontal disease have in common is that these conditions present mostly with a relatively mild phenotype and are slowly progressive and chronic in nature. Furthermore, the pathology is usually not clinically evident until adulthood and the diseases are relatively common (Loos *et al.* 2005, Tabor *et al.* 2002). Complex diseases are typically polygenic and might have multiple gene associations which individually have weak effects but when combined with each other and external influences, such as environmental factors, result in variable disease manifestation (Burgner *et al.* 2006, Kinane & Hart 2003, Risch 2000).

Genetic polymorphism can be described as a genome segment (locus), within or outside a gene, in which alternate forms (alleles) are present. In population genetics, variation is polymorphic if all alleles are found at frequencies > 1%. In clinical genetics, a polymorphism refers to any genetic variation not known to be direct cause of disease, in contrast to a mutation. Polymorphisms are considered under the hypothesis that they can affect the development of the disease only in the presence of an environmental risk factor (Malats & Calafell 2003).

The most common form of polymorphism is the single nucleotide polymorphism (SNP), which is a single base-pair change at a specific point in the genomic DNA (Burgner *et al.* 2006). The genome is composed of a linear strand of nucleotides in a double-stranded helical array. The linear sequence of nucleotides in one of the strands codes for amino acids in the form of triplet codons. Most allelic variants involve a change from one of the four nucleotides (adenine (A), thymine (T), cytosine (C), guanine (G)) to another (Kinane & Hart 2003). There are other forms of genetic variation including repeating sequence motifs (microsatellites and minisatellites), insertions and deletions, and differences in gene copy number, but the SNPs are the most common ones

(Burgner *et al.* 2006, Crawford *et al.* 2005). The human genome consists of approximately three billion base pairs and approximately ten million positions in this genetic code are polymorphic in the human population at a significant frequency (International HapMap Consortium *et al.* 2007, Xavier & Rioux 2008). These SNPs are thought to account for approximately 90% of human genetic variation. It is these common sequence differences that result in phenotypic differences, such as height, skin color and differential resistance to infection or disease. The challenge is to determine which of the common variants contribute to disease susceptibility (Xavier & Rioux 2008, Palmer & Cardon 2005). In addition, although the occurrence of SNPs across the genome is frequent, most SNPs (64% of all) are rare, with minor allele frequency of < 5% (Crawford *et al.* 2005).

Genetic polymorphisms have historically been used as genetic markers to locate disease-causing genes through linkage studies. Linkage is a tendency for genes and other genetic markers to be inherited together because of their location near each other on the same chromosome. Genetic linkage analysis is a method to localize genes and markers with respect to each other in the genome, based on recombination frequency (Burgner *et al.* 2006, Tabor *et al.* 2002, Crawford *et al.* 2005).

When linkage study designs were used to identify genes involved in the predisposition to more common diseases, success was limited (Xavier & Rioux 2008). To date, most studies of complex diseases are association studies. Until recently they have used a candidate gene approach, and they have been either population-based or case-control studies testing relatively small cohorts of patients with a small number of genetic variants (Burgner *et al.* 2006, Xavier & Rioux 2008). A candidate gene is a known gene suspected to be associated with the disease of interest on the basis of the biological function of its protein (Malats & Calafell 2003). The choice of the candidate genes may come from animal data, the results of whole genome studies, clinical data, or biological plausibility (Burgner *et al.* 2006, Tabor *et al.* 2002). Single nucleotide polymorphisms within candidate genes may be causally related to changes in protein expression, structure and function. These, in turn, may lead to variations in phenotypic expression (Takashiba & Naruishi 2006, Kinane & Hart 2003). Targeting of genes considered to be specifically related to inflammatory cell function and the regulation of the immune system in inflammatory diseases is a typical candidate gene study, which is based on prior knowledge of the disease in relation to its phenotype (Takashiba & Naruishi 2006).

Association studies on a small number of patients have the disadvantage that the findings may often be false positive or false negative, and the results obtained may thus be contradictory (Xavier & Rioux 2008). An increase in sample size (to several hundreds or thousands) would correct this tendency. Today, it is possible to probe the entire genome in genome-wide association studies (GWA). Specifically, the International HapMap Project has catalogued the haplotype patterns across the human genome by analyzing more than three million SNPs (International HapMap Consortium *et al.* 2007). At present, studies use up to 0.5 million of the approximately 11 million SNPs in the human genome (Xavier & Rioux 2008). Genome-wide association studies also have a high false discovery rate, but the incorrect results may have another explanation, i.e. technical problems. Replication in an independent population is also essential in the case of genome-wide association studies (Burgner *et al.* 2006).

2.3.2 Gene polymorphism in periodontal disease

There are several features of the host's innate immune system which may contribute to genetic susceptibility to aggressive forms of periodontitis. These conditions are typically inherited as simple Mendelian traits, are usually due to genetic alterations of a single gene locus and include epithelial, connective tissue, fibroblast and leukocyte defects (Kinane & Hart 2003, Hodge & Michalowicz 2001).

However, genetic research in the last few years has focused mainly on chronic periodontitis and gene polymorphisms that play roles in immunoregulation or metabolism. In this respect, gene polymorphisms associated with the production of cytokines, cell-surface receptors, enzymes and others that are related to antigen recognition have been studied (Yoshie *et al.* 2007).

Interleukin-1 gene polymorphism

Three *IL-1* genes are arranged in a cluster on human chromosome 2q13. Two of the genes, namely *IL-1A* and *IL-1B*, encode the cytokines IL-1 α and IL-1 β , respectively, while the third gene, known as *IL-1RN*, encodes the receptor antagonist (IL-1Ra) protein (Nicklin *et al.* 1994).

One of the most extensively studied cytokine gene polymorphisms originally identified by Kornman and co-workers, is a composite genotype that includes 2 rare polymorphisms in the cluster of *IL-1* genes (allele 2 of the *IL-1A* -889 plus

allele 2 of the *IL-1B* +3953) (Kornman *et al.* 1997). The first of these is at position -889 in *IL-1A* promoter, where the least common allele (allele 2) results from a C to T transition. The second is at position +3954 (formerly referred to as +3953 (Greenstein & Hart 2002)), of the *IL-1B* gene, where the least common allele (allele 2) also results from a C to T transition. When compared with the risk of genotype negatives, this periodontitis-associated genotype significantly increased the risk of periodontitis in non-smokers and distinguished individuals with severe periodontitis from those with mild disease (Kornman *et al.* 1997).

Since these first findings, the role of *IL-1* gene polymorphism in periodontal disease has been studied intensively, but the results have been controversial. Most of the studies have been cross-sectional, and environmental factors, such as smoking and presence of periodontal bacteria, have influenced the results (Yoshie *et al.* 2007, Taylor *et al.* 2004, Huynh-Ba *et al.* 2007). A case-control study of 132 patients with chronic periodontitis found that *IL-1A* +4845 and *IL-1B* +3954 composite genotype correlated with the severity of periodontitis and the antibody responses to periodontal microbiota, but did not distinguish between periodontitis patients and controls (Papapanou *et al.* 2001). As *IL1A* -889 is in complete linkage disequilibrium with *IL1A* +4845, analysis of either SNP provides the same genetic information (Kornman & di Giovine 1998). In another study (Laine *et al.* 2001) both smoking and the presence of *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* were taken into account and it was found that carriage of these rare alleles of the *IL-1* cluster genes was associated with severe adult periodontitis in the absence of the above risk factors. In a recent study (Ferreira *et al.* 2008), T-containing genotypes of *IL1B* +3954, as well as *P. gingivalis*, *T. forsythia*, and *T. denticola* were associated with higher IL-1 β levels and with higher values of clinical parameters of disease severity in non-smoking chronic periodontitis patients. Moreover, the risk genotype of *IL1B* +3954 and periodontopathogens were found to independently and additively modulate the levels of IL-1 β in the samples comprising gingival tissue.

Some studies have found the positive composite *IL-1* genotype (*IL-1A* +4845 and *IL-1B* +3954) to have some prognostic value for periodontal disease progression (Cullinan *et al.* 2001, Nieri *et al.* 2002, McGuire & Nunn 1999) or to be an indicator of periodontal disease deterioration (Lang *et al.* 2000, De Sanctis & Zucchelli 2000). A longitudinal study over a period of 5 years in 295 patients revealed a relationship between *IL-1* positive genotype and increasing probing depth in non-smokers older than 50 years. Moreover, subjects with *IL-1* positive genotype harboring *Porphyromonas gingivalis* had also increased pocket depths

compared with *IL-1* genotype negative subjects with a similar microbiological profile (Cullinan *et al.* 2001). However, several other studies have failed to provide any evidence for a contribution of *IL-1* genotype to periodontal disease progression and/or response to treatment (Konig *et al.* 2005, Weiss *et al.* 2004, Cortellini & Tonetti 2004, Christgau *et al.* 2003, Ehmke *et al.* 1999).

Interleukin-6 gene polymorphism

The *interleukin-6* gene was demonstrated to be localized in chromosome 7p21 (Bowcock *et al.* 1988). An association between *IL-6* gene polymorphism at position -174 and periodontal disease has been found in many studies (Trevilatto *et al.* 2003, D'Aiuto *et al.* 2004c, Brett *et al.* 2005, Nibali *et al.* 2007b), although the reports as to which genotype (GG or GG/CG) is involved in the risk of periodontal damage are conflicting. The GG genotype of the *IL-6* gene and susceptibility to chronic periodontitis has been found in a Caucasian population in Brazil (Trevilatto *et al.* 2003). *IL-6* -174 GG genotype has also been reported to be associated with the presence of pathogenic bacteria in the periodontal pockets of persons with aggressive periodontitis (Nibali *et al.* 2007b). Contradictory to the previous findings high serum levels of IL-6 in periodontitis patients have been associated with carriage of the C-allele (D'Aiuto *et al.* 2004c).

On the other hand, there are studies that suggest lower susceptibility to periodontal disease to be associated with protective function of allele C, whose presence may reduce IL-6 production (Trevilatto *et al.* 2003, Holla *et al.* 2004).

Tumor necrosis factor α (TNF α) gene polymorphism

The *tumor necrosis factor* gene is located in chromosome 6 within the major histocompatibility complex class III region (Wilson *et al.* 1997). Eight single nucleotide polymorphisms in the promoter region have been found (Yoshie *et al.* 2007), of which -308 locus G/A is one of the best described (Wilson *et al.* 1997, Abraham & Kroeger 1999) and also reported to be linked to chronic periodontitis susceptibility (Fassmann *et al.* 2003). The less common A allele is associated with high TNF- α production (Abraham & Kroeger 1999). On the other hand, lack of association between *TNF*-polymorphism and periodontitis has been consistently reported (Donati *et al.* 2005, Folwaczny *et al.* 2004c), also in studies where besides locus -308 G/A also other bi-allelic polymorphisms (-376 G/A, -238 G/A, +489 G/A (Craandijk *et al.* 2002), -238 G/A (Galbraith *et al.* 1998)) have been

investigated. In these studies, transition from G to A served as a genetic marker for the disease. The above-mentioned reports were for Caucasian populations. In a study made among Japanese populations, *TNF- α* -1031 T/C, -863 C/A or -857 C/T SNPs were associated with severe adult periodontitis (the latter bases are the variants) (Soga *et al.* 2003).

Interleukin-10 gene polymorphism

The gene encoding interleukin-10 was mapped to chromosome 1q31-32 (Kim *et al.* 1992). Three promoter single nucleotide polymorphisms have been described in this gene: -1087 G/A, -819 C/T and -592 C/A (Moore *et al.* 2001, Kube *et al.* 1995), and a number of studies have shown that allelic variation in the *interleukin-10* promoter is associated with a variety of diseases in which immune dysregulation contributes to the pathogenesis (Bidwell *et al.* 1999). The majority of studies looking at chronic periodontitis have shown no association (Brett *et al.* 2005, Yamazaki *et al.* 2001, Gonzales *et al.* 2002, Babel *et al.* 2006), with only a few showing an association (Berglundh *et al.* 2003, Scarel-Caminaga *et al.* 2004, Reichert *et al.* 2008). Concerning the risk genotype, the results have been conflicting, depending on ethnic origin as well as different degrees of disease severity. *IL-10* -1087 GG genotype was associated with severe periodontal disease in Swedish population (Berglundh *et al.* 2003), but could not be linked to periodontitis in Japan (Yamazaki *et al.* 2001) or Brazil, where the AA fraction was the largest in the population with periodontal disease (Scarel-Caminaga *et al.* 2004).

Except for one study, the design has been cross-sectional. In one recent longitudinal study the *IL-10* genotype (-592 and -1082) was shown to have a significant relationship with periodontal disease progression in Australian population, the main effect of haplotypes being protective (Cullinan *et al.* 2008).

Gene polymorphism in the innate immunity receptors

Two variants of the human *TLR4* gene that differ in the extracellular domain of the gene have been associated with increased risk of Gram-negative infections (Schroder & Schumann 2005). One form of the *TLR4* gene has an A to G substitution at nucleotide 896 downstream of the transcription start codon, which results in an aspartic acid to glycine substitution at position 299 of the amino acid sequence. This frequently affects synergistically with a second single nucleotide

change that results in a threonine being replaced by isoleucine at amino acid 399. There is some evidence that the Asp299Gly genotype might have a greater functional impact than the Thr399Ile genotype (Arbour *et al.* 2000). However, the results of the association studies between *TLR* polymorphisms and chronic periodontal disease have mostly been negative (Folwaczny *et al.* 2004b, Laine *et al.* 2005, James *et al.* 2007, Imamura *et al.* 2008, Fukusaki *et al.* 2007).

A single nucleotide polymorphism in the proximal *CD14* promoter at position -260 has been identified (Baldini *et al.* 1999). T allele carriage is reported to be connected to many inflammatory and systemic diseases, such as Crohn disease (Klein *et al.* 2002), ulcerative colitis (Obana *et al.* 2002), myocardial infarction (Hubacek *et al.* 1999, Morange *et al.* 2005) and carotid atherosclerosis (Giacconi *et al.* 2007). In relation to the pathogenesis of periodontal disease, the carriage of the TT genotype of *CD14* -260 has been associated with disease susceptibility (Laine *et al.* 2005, Holla *et al.* 2002, Yamazaki *et al.* 2003, Folwaczny *et al.* 2004a). On the other hand, the proportion of subjects carrying the TT genotype has been reported to be significantly smaller in the group with severe periodontitis than in the periodontally healthy group (Donati *et al.* 2005).

2.4 Periodontal host response and gene polymorphism in type 1 diabetes

Diabetes mellitus (DM) increases the risk of periodontal disease (Taylor & Borgnakke 2008, Southerland *et al.* 2006). The level of glycemic control (Lalla 2007, Lim *et al.* 2007, Tervonen & Oliver 1993), the presence of diabetic complications (Shultis *et al.* 2007, Karjalainen & Knuutila 1996) and disease duration (Tervonen *et al.* 2000, Firatli *et al.* 1996) are important determinants of this relationship. Pathogenetic mechanisms behind the increased severity of periodontal disease among diabetics include impaired function of immune cells, such as abnormalities of T-cell activation (Arif *et al.* 2004) and impairment of neutrophil adherence, chemotaxis, and phagocytosis (Mealey & Ocampo 2007, Mealey 1999). Alterations in collagen metabolism, bone formation (Lu *et al.* 2003, Gooch *et al.* 2000) and in inflammatory response to oral pathogens (Graves *et al.* 2005, Naguib *et al.* 2004) have been reported. In a hyperglycemic state an increased rate of apoptosis of the matrix-producing cells, such as fibroblasts and osteoblasts (Graves *et al.* 2007, Liu *et al.* 2004) has also been demonstrated.

Hyperglycemia has been shown to act deleteriously through a number of pathways, including aldose reductase pathway, advanced glycation end product

(AGE) pathway, reactive oxygen intermediate pathway, and protein kinase C pathway (King 2008, Scott & King 2004). All these pathways give rise to oxidant and inflammatory mediators that result in harmful effects both systemically and locally in the tissues. The effects include gene expression and/or protein function that contribute to cellular dysfunction and damage associated with micro-and macrovascular complications (King 2008).

The non-enzymatic glycosylation of long-lived proteins and lipids found in the blood and in the tissues, *i.e.* accumulation of advanced glycated end products (AGEs), also occurs in the periodontium. (Southerland *et al.* 2006, Mealey *et al.* 2006). AGEs in turn affect the secretion of cytokines and other inflammatory mediators by interacting with monocytic cell-surface receptors (RAGEs) (Southerland *et al.* 2006, Mealey & Ocampo 2007, Takeda *et al.* 2006). The AGE-RAGE interaction increases cellular oxidative stress and activates signaling molecules, such as nuclear factor-kappa B (NF- κ B), which alters the phenotype of monocytic cells and results in the increased production of proinflammatory cytokines, such as IL-1 β and TNF- α and IL-6 (King 2008, Mealey *et al.* 2006). Thus, local periodontal tissue destruction may be a consequence of an exaggerated monocytic inflammatory response induced by AGE accumulation resulting in exaggerated secretion of local and systemic inflammatory mediators (Southerland *et al.* 2006, Mealey & Ocampo 2007). It has been shown that the secretion of TNF- α , IL- β and PGE2 after *P. gingivalis* lipopolysaccharide (LPS) stimulation is higher by monocytes from insulin-dependent diabetes mellitus patients than by monocytes from systemically healthy controls. The release of the inflammatory mediators showed large inter-individual variation, with extremely high concentrations being secreted by some insulin-dependent diabetes mellitus patients. The abnormal monocytic response to LPS was associated with the diabetic state, a hyperresponsive monocytic phenotype and with more severe periodontal disease (Salvi *et al.* 1997a, Salvi *et al.* 1997b). In another study of diabetic subjects with periodontitis, those with hemoglobin A1c (HbA1c) levels > 8% had gingival crevice fluid levels of interleukin-1 β that were almost twice as high as in subjects whose hemoglobin A1c levels were < 8% (Engbretson *et al.* 2004). The same group also found that the plasma levels of TNF- α correlated with chronic periodontitis severity in type 2 diabetes patients (Engbretson *et al.* 2007). In diabetic mice, the blockade of RAGE has been found to diminish alveolar bone loss and to decrease the levels of matrix metalloproteinases, TNF- α , and IL-6 (Lalla *et al.* 2000).

Type 1 diabetes mellitus is a chronic autoimmune disease associated with multiple genetic and environmental risk factors. The role of cytokine gene polymorphisms has also been investigated in order to determine possible contributions to type 1 DM susceptibility. Only marginal or inconsistent evidence has been published to support the role of the cytokine polymorphisms in the pathogenesis of type 1 DM (Cooper *et al.* 2007, Kristiansen & Mandrup-Poulsen 2005, Kumar *et al.* 2007).

To date, only a few studies have been made to investigate whether gene polymorphisms of certain cytokine genes are associated with periodontal disease in diabetic patients. A recent cross-sectional study (Struch *et al.* 2008) reported an increase in the severity of periodontal disease along with increasing levels of HbA1c among type 2 diabetic subjects; furthermore, there was a significant association between T-bearing risk genotype of *IL-1A/1B* and periodontal disease in diabetic but not in non-diabetic subjects. Another study (Guzman *et al.* 2003) also found a trend suggesting that allele 1 at *IL-1B* was overrepresented among diabetic subjects with periodontal disease. On the other hand, *TNF- α* promoter polymorphism was not found to be associated with the presence of aggressive periodontitis in subjects with type 1 diabetes mellitus (Perez *et al.* 2004).

3 Aims of the study

The aim of this study was to evaluate whether the polymorphisms of the inflammation-associated cytokine and receptor molecule genes (*IL-1A* -889, *IL-1B* +3954, *IL-6* -174, *TNF- α* -308, *IL-10* -1082, *TLR4* +896, and *CD14* -260) contribute to the expression of chronic periodontitis. Another aim was to study possible systemic inflammatory responses evoked by chronic periodontitis. Further, we aimed to find out the significance of the above gene polymorphisms as determinants for the extent of periodontal disease in type 1 diabetes patients previously known to be at high risk for periodontal disease. More specifically the aims were:

- to compare the frequencies of the cytokine and receptor molecule genotypes in patients with moderate to severe chronic periodontitis with the corresponding frequencies in the reference subjects (I).
- to find out whether any associations exist between the extent of periodontal disease and the polymorphisms in the studied genes (I).
- to assess the significance of the extent of periodontal disease and the *IL-6* -174 or *CD14* -260 genotypes as determinants of respective serum IL-6 or sCD14 levels (II, III).
- to investigate whether cytokine and receptor molecule gene polymorphisms associate with the extent of periodontal disease in subjects with type 1 diabetes mellitus along with the diabetic status-associated factors (IV).

4 Material and methods

4.1 Study subjects

The study protocol was approved by the Ethical Committee of Oulu University Hospital, Oulu, Finland and the informed consent of all the subjects was obtained.

The study material consists of four study groups of Caucasian origin: the periodontitis group (n = 56), the control group (n = 28), the diabetes group (n = 80) and the reference group representing normal population (n = 178). The number of subjects in the periodontitis group varies between the papers, because all data were not available for all subjects.

Table 1. Characteristics of the study groups.

	N (female / male)	Age ¹ (years)	Smoking (no / yes)	Pocket depth ² ≥ 4mm	Attachment loss ² ≥ 4mm
Periodontitis group					
Paper I	51 34/17	42.9 ± 9.3	15/36	51.8 ± 23.9	47.4 ± 28.4
Paper II	52 35/17	43.2 ± 9.3	18/34	51.5 ± 23.4	46.3 ± 28.4
Paper III	56 37/19	43.2 ± 9.2	20/36	51.3 ± 23.1	45.2 ± 28.3
Control group	28 18/10	40.0 ± 10.7	18/10	0.9 ± 2.16	0.51 ± 1.5
Diabetes group	80 46/34	38.9 ± 12.3	56/24	24.2 ± 20.3	13.8 ± 24.0
Reference group	178 122/56	39.4 ± 13.4			

¹ Mean ± standard deviation ² Mean percentage of sites per subject ± standard deviation

A clinical periodontal examination was performed on the subjects in the periodontitis, the control and the diabetes group. Subjects needing prophylactic antibiotic medication in association with periodontal probing as well as those with antibiotics during the past four months were excluded from all the clinically examined groups.

The subjects in the periodontitis group (Papers I, II, III, Table 1) had moderate to severe chronic periodontitis. They were originally referred to periodontal specialist therapy at the Specialist Dental Health Care Unit, City of Oulu, Oulu, Finland, and volunteered to participate in the study. The patients were in principle periodontally untreated. Subjects with rheumatoid arthritis, diabetes mellitus, cardiovascular diseases, asthma or immunosuppressive medication were excluded from this group.

The control group (Paper III, Table 1) consisted of systemically healthy subjects matched with the periodontitis subjects for age and gender. In these subjects probing pocket depth (PD) \geq 4mm was detected in 0.9% and attachment level (AL) \geq 4mm in 0.5% of the total of 3,068 sites measured.

All the subjects in the diabetes group (Paper IV, Table 1) had type 1 diabetes mellitus and were mainly recruited from a primary health care diabetes unit. A few patients were recruited from The Clinic of Internal Medicine, Oulu University Hospital, Oulu, Finland.

The reference group (Papers I and IV) representing normal population consisted of university staff and students, who were informed of the study and volunteered to give their blood samples to be used as reference samples (Karhukorpi *et al.* 2002) in studies where genotype frequencies of different groups would be compared (Papers I and IV). No data concerning the medical health or periodontal health status of the reference subjects were available.

4.2 Clinical and radiographic periodontal variables, smoking habits and body mass index

All the subjects were examined by the same periodontal specialist. Periodontal variables were recorded at four sites (mesiobuccal, midbuccal, distobuccal and midlingual) of all teeth excluding third molars. The presence of visible plaque was assessed after gentle drying with air corresponding to the criteria of scores 2 and 3 of the plaque index (Silness & Loe 1964) (Paper IV). Probing pocket depth (PD) was measured using a ball-pointed periodontal probe with 2 mm graduations from the gingival margin to the base of the crevice/pocket (Papers I–IV). Bleeding on probing (BOP) was scored positive if a site bled within 20–30 seconds after probing (Paper IV). Periodontal attachment level (AL) was measured from the cementoenamel junction to the base of the crevice/pocket (Papers I–IV). Alveolar bone level (BL) was measured by one examiner from orthopantomograms at the mesial and distal sites of the teeth using a Dimaxis

Planmega application (Papers I, II, and III). The actual distances measured from the cementoenamel junction to the level of the alveolar ridge where the periodontal ligament space started to be of uniform width were measured from the scanned orthopantomograms and then automatically calibrated to millimeters by the application. Any site where the cementoenamel junction or the alveolar crest could not be identified was excluded as non-measurable. Duplicate measurements of the distance were made at each site, and if the difference between these two measurements was ≤ 1 mm, the first value was used in the analyses. If the difference was > 1.0 mm, a third measurement was made and the one of the first two measurements that was closer to the third measurement was used.

As regards plaque and BOP, the mean percentages of affected sites per subject were used in statistical analyses. The severity/extent of periodontal disease was expressed as the mean percentages of affected sites per subject using two different threshold values for each variable (≥ 4 mm and ≥ 6 mm for PD, ≥ 4 mm and ≥ 6 mm for AL and ≥ 6 mm and ≥ 8 mm for BL).

Smoking habits, i.e. the number of cigarettes smoked per day, were obtained by interviewing the subjects in association with the clinical examination. The subjects' body mass indices (BMI, weight divided by the square of the height, kg/m^2) were also recorded.

4.3 Analyses of cytokine and receptor molecule gene polymorphism

Cytokine and receptor gene polymorphisms (Papers I–IV) were tested in order to identify single nucleotide polymorphisms (SNPs) using PCR (polymerase chain reaction) with RFLP (restriction fragment length polymorphism) (*IL-6* -174, *TNF- α* -308, *IL-1A* -889, *IL-1B* +3954, and *TLR4* +896) or allele-specific primers (*CD14* -260 and *IL-10* -1082). The primers and methods were taken from the literature and/or developed by our own group: *CD14* -260 earlier known as *CD14* -159 (Hubacek *et al.* 1999, Karhukorpi *et al.* 2002), *IL-6* -174 (Fishman *et al.* 1998), *TNF- α* -308 (Ozen *et al.* 2002), *IL-10* -1082 (Karhukorpi & Karttunen 2001), *IL-1A* -889 (McDowell *et al.* 1995), *IL-1B* +3954 (Luomala *et al.* 2001), and *TLR4* +896 (Lorenz *et al.* 2001). PCR (polymerase chain reaction) conditions were optimized by comparing various annealing temperatures. The genotypes in each cytokine gene were grouped according to their known biological significance, so that in a pro-inflammatory cytokine, a genotype/genotypes known to produce higher levels of the cytokine (positive genotype) were estimated

separately from the one(s) known to be associated with lower secretion. Genotypes of *CD14* -260 containing T allele are known to be associated with a higher soluble amount of CD14 (Baldini *et al.* 1999, Karhukorpi *et al.* 2002). The G-containing genotype of *IL-6* -174 is known to be associated with higher IL-6 secretion (Fishman *et al.* 1998, Hulkkonen *et al.* 2001). We chose to analyze the data using GG vs. GC/CC grouping. Genotypes of *TNF- α* -308 containing A allele(s) are known to be associated with higher TNF- α secretion (McGuire *et al.* 1994). There is some indication that the genotypes of *IL-1A* -889 and *IL-1B* +3954 containing T allele are associated with higher IL-1 secretion (Taylor *et al.* 2004). A genotype of a regulatory cytokine IL-10 associated with lower secretion (*IL-10* -1082, AA) was analyzed separately (Turner *et al.* 1997). For *TLR4* +896 polymorphism, a mutant genotype containing G is associated with a weaker receptor action (Arbour *et al.* 2000) with an attenuated response to LPS.

4.4 Laboratory analyses of serum

The blood samples were obtained by venipuncture (2x10ml). Serum IL-6 levels (pg/ml) (Paper II) were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) Quantikine HS Immunoassay kit (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) and serum sCD14 concentration (μ g/ml) (Paper III) by sensitive Human sCD14 ELISA test kit, (Hycult biotechnology, Uden, Netherlands).

4.5 Determination of diabetes status

The glycosylated hemoglobin value (HbA1c) of each subject in the diabetes group was tested at the time of the clinical examination. The previous HbA1c values were retrieved from patient records. The mean value of the HbA1c levels over the past three years (3-yr HbA1c) was calculated to indicate the long-term control of diabetes mellitus (on the average 6.6 measurements/subject). While a laboratory immunoassay method was used to analyze the HbA1c level at the time of the clinical examination, the three-year values were based both on a laboratory immunoassay and on measurements using a DCA device (DCA, Siemens, Eschorn, Germany) in the diabetes unit. Along with the duration of diabetes mellitus, diabetic complications were registered from patient records and categorized as follows: presence or absence of retinopathy (proliferative or

nonproliferative stage), nephropathy (micro- or macroalbuminurea), peripheral neuropathy and macrovascular complications (stroke or myocardial infarction).

4.6 Statistical analyses

The between-group differences in the distributions of the subjects were analyzed using either Fisher's exact test or the Chi-square test. Significances of the differences in the mean values were determined using Student's t-test or in case of skewed distributions, the non-parametric Mann-Whitney test. Associations between the extent of periodontal disease and the cytokine and receptor molecule genotypes were analyzed using linear models. Adjustments were made for gender and smoking. The associations between the serum IL-6 and sCD14 levels, on the one hand, and the extent of periodontal disease and the respective genotypes, on the other hand, were also determined using linear models adjusting for age, gender, smoking and BMI.

All calculations were performed using a statistical software package (SPSS, version 16.0, SPSS Inc., Chicago, IL, USA).

5 Results

5.1 Associations between cytokine and receptor gene polymorphisms and the extent of periodontal disease in the periodontitis group (I)

5.1.1 SNPs

There were no statistically significant differences in the frequencies of the cytokine genotypes between the subjects with chronic periodontitis and the reference subjects representing a sample of normal population, when any of the SNPs (*CD14* -260, *IL-6* -174, *TNF- α* -308, *IL-10* -1082, *IL-1A* -889, *IL-1B* +3954 and *TLR4* +896) was considered.

Of all SNPs studied, *CD14* -260 and *IL-6* -174 polymorphisms were most significantly associated with the severity of periodontal disease. When compared to the rest of the periodontitis group, the subjects with the T-containing genotype of *CD14* -260 had significantly more sites with PD \geq 4mm ($p = 0.044$) and BL \geq 6mm ($p = 0.022$), and those with the GG genotype of the *IL-6* -174 had a higher extent of AL \geq 4mm ($p = 0.056$) and BL \geq 6mm ($p = 0.058$). Subjects carrying the GG genotype of *TNF- α* -308 had significantly more sites with PD \geq 4mm than subjects carrying the A-containing genotype ($p = 0.048$) (Table 2).

As regards the extent of advanced periodontal disease, the subjects carrying the T-containing genotype of *CD 14* -260 or GG genotype of *IL-6* -174 had a higher extent of sites with PD \geq 6mm ($p = 0.053$, $p = 0.06$, respectively), AL \geq 6mm ($p = 0.045$, $p = 0.048$, respectively) and BL \geq 8mm ($p = 0.065$, $p = 0.089$, respectively) (Fig 1).

After adjusting for smoking and gender, *CD14* -260 genotype associated significantly with the percentage of sites with PD \geq 6mm ($p = 0.032$), AL \geq 6mm ($p = 0.024$) and BL \geq 8mm ($p = 0.045$). After the same adjustments, *IL-6* genotype was significantly associated with the frequency of PD \geq 6mm ($p = 0.01$). No interaction between these two genotypes was found in the multivariate model (Table 3).

Table 2. Percentages of sites with PD ≥ 4mm, AL ≥ 4mm and BL ≥ 6mm by genotype.

Genotype	N	PD ≥ 4mm ¹	AL ≥ 4mm ¹	BL ≥ 6mm ¹
<i>CD14 -260</i>				
TT/CT	24	58.9 ± 26.6	54.3 ± 31.4	39.7 ± 30.1
CC	27	45.5 ± 19.5	41.2 ± 24.4	22.7 ± 20.6
<i>p</i> -value		0.044	0.102	0.022
<i>IL-6 -174</i>				
GG	11	62.1 ± 27.4	61.8 ± 30.6	44.2 ± 31.5
CC/CG	40	49.0 ± 22.4	43.4 ± 26.8	27.0 ± 24.3
<i>p</i> -value		0.107	0.056	0.058
<i>TNF-α -308</i>				
AG/AA	16	42.1 ± 21.9	40.1 ± 24.2	23.1 ± 23.9
GG	35	56.2 ± 23.7	50.7 ± 29.8	34.2 ± 27.4
<i>p</i> -value		0.048	0.218	0.169
<i>IL-10 -1082</i>				
AA	19	57.3 ± 23.4	53.0 ± 29.2	27.7 ± 23.6
AG/GG	32	48.5 ± 23.9	44.1 ± 27.9	35.8 ± 31.2
<i>p</i> -value		0.207	0.283	0.300
<i>IL-1A -889</i>				
TT/CT	28	50.7 ± 26.4	44.2 ± 30.7	30.0 ± 30.3
CC	23	53.1 ± 20.9	51.3 ± 25.4	31.5 ± 22.1
<i>p</i> -value		0.730	0.381	0.841
<i>IL-1B +3954</i>				
TT/CT	25	52.7 ± 24.8	44.4 ± 31.7	30.8 ± 30.0
CC	26	50.9 ± 23.4	50.2 ± 25.1	30.6 ± 23.7
<i>p</i> -value		0.787	0.472	0.976
<i>TLR-4 +896</i>				
AG/GG	13	49.1 ± 25.1	45.0 ± 30.3	24.3 ± 23.7
AA	38	52.7 ± 23.5	48.2 ± 28.1	32.9 ± 27.6
<i>p</i> -value		0.639	0.727	0.325
<i>CD14 -260 CT/TT +</i>				
<i>IL-6 -174 GG</i>	8	67.9 ± 29.2	70.1 ± 31.2	51.2 ± 33.5
Any other combination	43	48.8 ± 21.8	43.2 ± 26.1	26.9 ± 23.8
<i>p</i> -value		0.036	0.012	0.016
<i>CD14 -260 CT/TT +</i>				
<i>TLR4 +896 AG/GG</i>	7	52.1 ± 32.9	52.2 ± 33.3	30.2 ± 27.1
Any other combination	44	51.7 ± 22.6	46.6 ± 27.9	33.7 ± 25.3
<i>p</i> -value		0.966	0.635	0.582

In the first column the positive genotype is bolded.

Statistical significances were tested using Student's *t*-test.

¹ Mean percentage of sites ± standard deviation

AL, attachment level; BL, bone level; PD, pocket depth

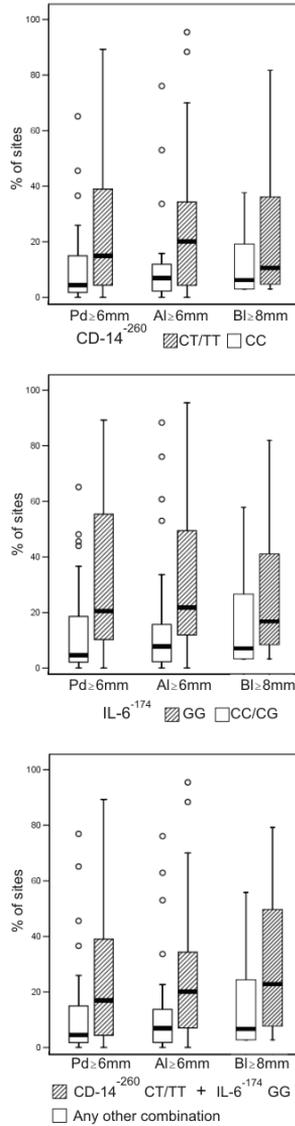


Fig. 1. Box-plots presenting the percentages of sites with PD ≥ 6mm, AL ≥ 6mm and BL ≥ 8mm by genotype. Significances of differences between genotypes using the non-parametric Mann-Whitney test: CD14⁻²⁶⁰: PD ≥ 6mm, p = 0.053, AL ≥ 6mm, p = 0.045, BL ≥ 8mm, p = 0.065. IL-6⁻¹⁷⁴: PD ≥ 6mm, 0.06, AL ≥ 6mm, p = 0.048, BL ≥ 8mm, p = 0.089. CD14⁻²⁶⁰/IL-6⁻¹⁷⁴: PD ≥ 6mm, p = 0.014, AL ≥ 6mm, p = 0.009, BL ≥ 8mm, p = 0.056.

Table 3. Parameter estimates of the analyses of variance using percentages of sites with PD \geq 6mm, AL \geq 6mm and BL \geq 8mm as dependent variable.

Genotype	PD \geq 6mm ¹			AL \geq 6mm ¹			BL \geq 8mm ¹		
	β	95% CI for β	<i>p</i>	β	95% CI for β	<i>p</i>	β	95% CI for β	<i>p</i>
CD-14 CT+TT	13.1	1.2-25.1	0.032	15.1	2.1-28.1	0.024	10.6	0.2-20.9	0.045
CD-14 CC	0			0			0		
IL-6 GG	19.3	4.9-33.6	0.010	15.3	0.9-31.6	0.064	11.1	-1.7-23.9	0.088
IL-6 CC+CG	0			0			0		

In the first column the positive genotype is bolded.

Adjusted for gender and smoking.

¹Percentages of affected sites.

AL, attachment level; BL, bone level; PD, pocket depth; β , standardized coefficient; CI, confidence interval.

5.1.2 Combination of SNPs

When *CD14* -260 and *IL-6* -174 genotypes were combined, those eight subjects who carried positive genotypes of both had significantly higher percentages of sites with PD \geq 4mm ($p = 0.036$), AL \geq 4mm ($p = 0.012$) and BL \geq 6mm ($p = 0.016$) than the rest of the periodontitis group (Table 2).

In the subjects carrying positive genotypes of both *CD 14* -260 and *IL-6* -174 the extent of sites with PD \geq 6mm ($p = 0.014$) was approximately three times and the extent of AL \geq 6mm ($p = 0.009$) and BL \geq 8mm ($p = 0.056$) more than 2.5 times that observed in the rest of the periodontitis group (Fig 1).

5.2 Associations between serum IL-6 level and *IL-6* -174 genotype and the extent of periodontal disease in the periodontitis group (II)

The mean concentration of IL-6 (\pm s.d.) in serum was 1.6 pg/mL (\pm 1.5, median 1.4 pg/mL). In subjects carrying the GG genotype the serum concentration of IL-6 was higher (2.8 ± 2.6 pg/mL) than in those carrying the CG/CC genotype (1.4 ± 0.9 pg/mL).

BMI-, gender-, age- and smoking-adjusted associations between the percentages of sites with PD \geq 6mm, AL \geq 6mm and BL \geq 8mm and serum IL-6 level were statistically significant. *IL-6* -174 genotype was also a significant determinant of the serum IL-6 level as well as the BMI (Table 4).

Table 4. BMI-, gender-, age- and smoking-adjusted associations between serum IL-6 level (the outcome variable) and *IL-6* -174 genotype and the extent of periodontal disease (PD ≥ 6mm, AL ≥ 6mm and BL ≥ 8mm in separate models).

	B	95% CI for β	<i>p</i> -value	<i>R</i> ²
PD ≥ 6mm	0.033	0.017–0.049	<0.001	
<i>IL-6</i> -174 genotype ¹	0.761	-0.099–1.621	0.081	
BMI	0.136	0.038–0.233	0.007	
Gender ²	0.799	0.056–1.541	0.036	
Age	0.014	-0.024–0.053	0.449	
Smoking ³	0.238	0.056–1.541	0.491	0.496
AL ≥ 6mm	0.025	0.010–0.041	0.002	
<i>IL-6</i> -174 genotype ¹	0.985	0.102–1.868	0.030	
BMI	0.144	0.041–0.247	0.007	
Gender ²	0.622	-0.161–1.404	0.117	
Age	0.002	-0.039–0.043	0.916	
Smoking ³	0.106	-630–0.843	0.773	0.439
BL ≥ 8mm	0.042	0.023–0.061	<0.001	
<i>IL-6</i> -174 genotype ¹	0.900	0.078–1.721	0.033	
BMI	0.146	0.050–0.242	0.004	
Gender ²	0.856	0.125–1.587	0.023	
Age	0.018	-0.021–0.058	0.353	
Smoking ³	0.047	-0.640–0.735	0.891	0.513

Continuing variables: percentages of sites with PD ≥ 6mm, AL ≥ 6mm and BL ≥ 8mm, BMI (kg/m²) and age (years). ¹*IL-6* genotype: GG compared with GC/CC. ²Gender: females compared with males.

³Smoking: smokers compared with non-smokers. BMI, body mass index; PD, pocket depth; AL, attachment level; BL, bone level; CI, confidence interval; β , standardized coefficient; *R*², coefficient of determination.

In subsequent models, possible interaction between the *IL-6* -174 genotype and the extent of periodontal disease (PD ≥ 6mm, AL ≥ 6mm and BL ≥ 8mm) was evaluated. Significant interaction was found between the extent of periodontal disease and the *IL-6* genotype. In these analyses, the extent of advanced periodontal disease (PD ≥ 6mm, AL ≥ 6mm and BL ≥ 8mm) remained a significant independent variable associated with serum IL-6 level whereas the *IL-6* genotype did not.

5.3 Serum sCD14, polymorphism of *CD14* -260 and periodontal infection (III)

The mean concentration of sCD14 (\pm s.d.) in serum was significantly higher in the patients with periodontitis than in the control subjects ($4.9 \pm 1.2 \mu\text{g/mL}$ vs. $3.8 \pm 7.3 \mu\text{g/mL}$, $p < 0.001$). Irrespective of their periodontal health status, the subjects carrying the CT/TT genotype presented significantly higher sCD14 levels than did those carrying the CC genotype ($4.9 \pm 1.4 \mu\text{g/mL}$ vs. $4.3 \pm 10.0 \mu\text{g/mL}$, $p = 0.019$). The differences in the concentrations of sCD14 by genotype after stratification by periodontal health status (periodontitis group vs. control group) were statistically significant ($p = 0.03$) only in the control subjects (Fig. 2).

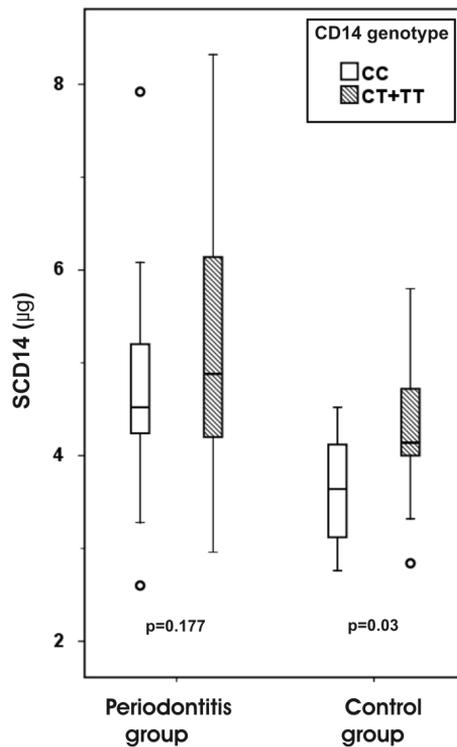


Fig. 2. Concentrations of sCD14 ($\mu\text{g/ml}$) by *CD14* -260 genotype in subjects with periodontitis and in control subjects.

Associations of serum sCD14 concentration with periodontal health status and the *CD14* -260 genotype were analyzed by means of linear regression analysis, adjusting for age, gender, smoking and BMI. Both the *CD14* -260 genotype and the subject group (periodontitis vs. control) were significant determinants for serum sCD14 concentration (Table 5).

Table 5. Age-, gender-, smoking- and BMI¹-adjusted associations between serum sCD14 level (the outcome variable) and *CD14* -260 genotype and subject group.

	B	95% CI for β	p-value
<i>CD14</i> -260 genotype ²	486.8	10.8–962.8	0.045
Subject group ³	887.6	354.4–1420.8	0.001

¹ Body mass index (kg/m²). ² *CD14* -260 genotype: CT/TT compared with CC. ³ Subject group: Periodontitis subjects compared with controls. β , standardized coefficient; CI confidence interval.

After adjustments for age, gender, smoking and BMI, the extent of advanced periodontal disease as indicated by the percentages of sites with AL \geq 6mm and BL \geq 8mm associated significantly with serum sCD14 concentration in subjects with periodontitis (Table 6).

Table 6. Age-, gender-, smoking- and BMI¹-adjusted associations between serum sCD14 levels (the outcome variable) and the extent of periodontal disease.

	B	95% CI for β	p-value
PD \geq 6mm ²	14.6	-0.7–30.0	0.062
AL \geq 6mm ²	21.4	8.0–34.8	0.002
BL \geq 8mm ²	24.6	7.1–42.1	0.007

¹ Body mass index (kg/m²). ² Percentages of affected sites.

PD, pocket depth; AL, attachment level; BL, bone level; β , standardized coefficient; CI, confidence interval.

5.4 Association between *IL-6* -174 genotype and the extent of periodontal disease in type 1 diabetic subjects (IV)

No statistically significant differences were found in the frequencies of the cytokine genotypes between the diabetic patients and the reference subjects when any of the SNPs (*CD14* -260, *IL-6* -174, *TNF- α* -308, *IL-10* -1082, *IL-1A* -889, *IL-1B* +3954 and *TLR4* +896) was considered.

After evaluating unadjusted associations between the extent of periodontal disease and explanatory variables, the mean value of glycosylated hemoglobin

over the past three years (3yr-HbA1c), duration of DM, and the *IL-6* -174 genotype were found to be significantly associated with the extent of BOP and $PD \geq 4\text{mm}$.

In the subsequent regression analysis adjustments were made for duration of diabetes, plaque, age, smoking and gender. In addition to plaque, the *IL-6* -174 genotype and 3yr-HbA1c were statistically significantly associated with BOP and $PD \geq 4\text{mm}$ (Table 7).

Table 7. Plaque-, age-, duration of DM-, smoking- and gender-adjusted associations between BOP and $PD \geq 4\text{mm}$ and 3-yr HbA1c and *IL-6* -174 genotype.

	BOP ¹			$PD \geq 4\text{mm}$ ¹		
	β	95%CI for β	p-value	β	95%CI for β	p-value
3-yr HbA1c ²	3.24	0.94–5.55	0.007	3.64	0.94–6.35	0.009
<i>IL-6</i> -174 genotype ³	9.61	2.46–16.76	0.009	11.28	2.92–19.64	0.009
Plaque ¹	0.34	0.21–0.47	<0.001	0.39	0.24–0.54	<0.001
Age	0.16	-0.11–0.44	0.237	0.18	-0.14–0.50	0.279
Duration of DM	0.09	-0.20–0.37	0.546	0.28	-0.05–0.61	0.098
Smoking ⁴	5.66	-0.38–11.70	0.066	11.17	4.02–18.24	0.002
Gender ⁵	4.32	-1.71–10.34	0.157	-0.08	-7.13–6.97	0.982

¹Percentages of affected sites.

Continuing variables: ²Mean value of glycosylated hemoglobin over the past three years, percentages of sites with plaque, age (years), duration of diabetes mellitus (years).

³*IL-6* genotype: GG compared with GC/CC.

⁴Smoking: smokers compared with non-smokers.

⁵Gender: males compared with females.

DM, diabetes mellitus; BOP, bleeding on probing; PD, pocket depth; HbA1c, glycosylated hemoglobin; β , standardized coefficient; CI, confidence interval

After stratification by *IL-6* -174 genotype the associations between BOP and $PPD \geq 4\text{mm}$ and 3yr-HbA1c levels remained significant in subjects carrying the GC/CC but not the GG genotype (Table 8).

Table 8. Plaque-adjusted associations between BOP and PD \geq 4mm and 3yr-HbA1c separately in subjects with the GG and CG/CC *IL-6* -174 genotype.

	BOP ²				PD \geq 4mm ²			
	β	95% CI for β	p	R ²	β	95% CI for β	p	R ²
GG genotype								
3yr-HbA1c ¹	1.09	-3.26–5.43	0.600	0.531	2.99	-6.16–12.14	0.495	0.437
GC/CC genotype								
3yr-HbA1c ¹	4.31	1.62–6.99	0.002	0.334	4.54	1.74–7.34	0.002	0.355

¹Mean value of glycosylated hemoglobin over the past three years. ²Percentages of affected sites.
 BOP, bleeding on probing; PD, pocket depth; CI, confidence interval; β , standardized coefficient; R², coefficient of determination.

6 Discussion

6.1 Study design and variables

Periodontitis is one of the most common chronic diseases in adults. Although initiated by bacteria, the host immune responses and variations in susceptibility to periodontal disease play important roles in the pathogenesis. Most human diseases and individual variations in the host immune responses have genetic components in them (Yoshie *et al.* 2007). A number of scientific papers support the role of genes in host responses and in the progression of periodontal disease as well (Takashiba & Naruishi 2006, Yoshie *et al.* 2007, Loos *et al.* 2005). In Finland, the role of genetic variation, gene polymorphism, has been studied in relation to several inflammatory diseases, of which atherosclerosis (Hulkkonen *et al.* 2008), *Helicobacter pylori* infection (Karhukorpi *et al.* 2002), Sjögren's syndrome (Hulkkonen *et al.* 2001) and multiple sclerosis (Luomala *et al.* 2001) can be mentioned. This study is the first one evaluating the associations between cytokine and receptor molecule gene polymorphisms and periodontal disease in study groups consisting of Finnish population. We also studied the systemic inflammatory response evoked by chronic periodontitis by assessing actual serum levels of IL-6 and sCD14 and relating them to the severity of periodontal disease and *IL-6* -174 and *CD14* -260 genotypes, respectively.

Of the four study groups, the periodontitis group comprised patients with moderate to severe chronic periodontitis. A group of subjects with type 1 diabetes mellitus and with varying extent of periodontal disease was also recruited. A reference group representing normal population of Caucasian origin from the same area was used to compare genotype frequencies between the study groups. This reference group has also been used in *Helicobacter pylori* studies (Karhukorpi *et al.* 2002) and in reports concerning genetic variations in intervertebral disc disease (Noponen-Hietala *et al.* 2005). Finally, systemically healthy subjects matched for age and gender served as controls to the periodontitis subjects. Using the above groups we were able to study whether any accumulation of risk genotypes occurred in the periodontitis group. Inside the periodontitis group we could relate the severity/extent of periodontal disease to various genotypes and to the serum levels of sCD14 and IL-6. Diabetes is known to increase the risk for periodontitis (Taylor & Borgnakke 2008, Southerland *et al.*

2006), but so far no studies are available on the possible regulative role of gene polymorphism in periodontal infection in subjects with type 1 DM.

From a genetic point of view the present study is an association study, where cytokine and receptor molecule genes and their polymorphic variants serve as candidate genes. In clinical genetics the results of association studies are often criticized because of small sample sizes and low statistical power (Burgner *et al.* 2006). Experts in genetic epidemiology, however, currently favor association study designs in complex diseases that do not need sampling of DNA from families and in which the disease phenotype also depends on an environmental impact (Burgner *et al.* 2006, Palmer & Cardon 2005, Stuber *et al.* 2005). Still, association studies concerning complex diseases easily encounter problems, because genomic markers often show very low and insignificant relative risks when associated with the disease. Achievement of adequate statistical power in these studies is often impossible, because the required case numbers increase exponentially with decreasing relative risk (Stuber *et al.* 2005).

The number of meta-analyses of genetic studies is continuously rising (Nikolopoulos *et al.* 2008) and therefore studies made in smaller sample groups, like the ones in our study, may not be recommended, albeit justified. Combining a few genetic variants, even with moderate effects, may improve our ability to analyze risks and the utility of genetic research concerning periodontitis as well (Nikolopoulos *et al.* 2008, Ioannidis *et al.* 2006). The possibility of genotyping hundreds of thousands of SNPs using various microarray platforms and the availability of haplotype maps of the genome that can guide more efficient SNP selection will change the genetic analysis of common diseases (Palmer & Cardon 2005). On the other hand, when gene polymorphisms are studied in relation to disease severity, data obtained also from smaller study groups will add useful information concerning the genetic variation of the disease.

The cytokines and their gene polymorphisms analyzed (*CD14* -260, *IL-6* -174, *TNF- α* -308, *IL-10* -1082, *IL-1A* -889, *IL-1B* +3954, and *TLR4* +896) were chosen because of their known biological significance in the pathogenesis of inflammatory and infectious diseases including periodontitis. The systemic inflammatory response was assessed as the serum levels of sCD14 and IL-6, because polymorphisms in genes coding them were shown to be significantly associated with the extent of periodontal disease in the study subjects. Cytokines do not act in isolation but form a network of high complexity. Together with their receptors, they are under tight biological control, including positive and negative feedback by the cytokines themselves (Gorska *et al.* 2003). Hence, when

correlating the serum levels of cytokines and the severity of the disease, the interaction between various cytokines and other unknown modifying factors should be kept in mind. Overall, due to the cross-sectional setting of this study, one must be careful in drawing definite conclusions on the causality between different variables, such as periodontal infection and serum levels of cytokines.

The outcome variables used in this study included various measures of the extent of periodontal disease, namely bleeding on probing, pocket depth and attachment and bone level. These parameters complete each other: bleeding on probing and pocket depth represent the state of periodontal infection at the moment, whereas the magnitude of periodontal attachment and bone loss are most likely indicative of a lifelong predisposition to periodontal disease.

Confounding and modifying factors were considered in analyzing the strength of various associations. This was performed by data stratification as well as using multivariate analyses with or without interaction terms. In that regard, the gender and the age of the subjects were analyzed. We also considered body mass index (BMI), and of environmental risk factors, smoking. Cigarette smoking is a well-established risk factor for periodontitis and associated with a two- to eight-fold risk for periodontal attachment and/or bone loss, depending on the definition of disease severity and smoking dose (Johnson & Guthmiller 2007). Smoking was common among the subjects, but did not overpower the influence of the genotype. In fact, it has been suggested that smoking and a positive genotype may act in synergy (Meisel *et al.* 2002). In the present study the subjects were categorized as non-smokers and smokers. We were not able to categorize the subjects by the numbers of cigarettes smoked per day, because the numbers of the subjects in the subgroups were too small for statistical analyses.

6.2 Cytokine and receptor molecule gene polymorphism in chronic periodontitis

Genetic variation that results in altered structure or expression of a cytokine can have evident pathological consequences, potentially leading to increased risk of infection, a number of chronic diseases or altered outcome of acute disorders (Smith & Humphries 2009). The biological basis for the association of cytokine gene polymorphism and periodontal disease is that carriage of certain alleles of a cytokine gene is related to increased production of the cytokine. A hyperresponsive individual may secrete increased amounts of inflammatory

mediators and generate a risk for the initiation and progression of periodontal disease.

The main finding of this study was that inside the periodontitis group, carriage of the GG genotype of *IL-6* -174 and the T-containing genotype of *CD14* -260 were separately associated with the extent periodontal disease. The genotype frequencies were however similar between the periodontitis and reference subjects. Previously, carriers of the GG genotype of *IL-6* -174 have been shown to have a higher risk for worsening of many systemic diseases, such as ischemic stroke or atherosclerosis (Giacconi *et al.* 2008). An increase in the frequency of the -174 G allele has been found in periodontitis patients compared with controls of unknown periodontal status (Brett *et al.* 2005). In agreement with our results an association between the GG genotype of the *IL-6* -174 gene and susceptibility to chronic periodontitis has been reported in a Caucasian population in Brazil (Trevilatto *et al.* 2003). The GG genotype of *IL-6* -174 has also been found to be associated with the presence of pathogenic bacteria, such as *A.actinomycetemcomitans* and *P.gingivalis*, indicating that this polymorphism may be an important variant in determining susceptibility to colonization with periodontopathogenic bacteria (Nibali *et al.* 2007b).

CD14 -260 C to T polymorphism has been reported to be associated with several inflammatory diseases, such as asthma (Garty *et al.* 2000, Martin *et al.* 2006) and cardiovascular events (Giacconi *et al.* 2008), in which the genetically determined increased inflammatory responses against pathogens or antigens are suggested to play a role. In line with the present results, increased frequencies of -159 TT homozygotes have previously been found among subjects with severe periodontitis when compared with a subgroup with moderate periodontitis (Holla *et al.* 2002) or periodontally healthy controls (Laine *et al.* 2005). Evidently the carriage of the T allele is associated with higher activation of the immune response to the lipopolysaccharide/lipopolysaccharide binding protein (LPS/LBP) complex, and thus to increased progression of periodontal disease. When compared with the CC or CT genotypes, the homozygotes of the allele 159 T have been observed to express a higher density of the membrane-bound mCD14 receptors on monocytes (Le Van *et al.* 2001, Hubacek *et al.* 1999, Yamazaki *et al.* 2003) and to produce higher amounts of TNF- α after stimulation by *P. gingivalis* LPS (Yamazaki *et al.* 2003).

It was also noted that the highest extent of periodontitis was observed in subjects carrying a positive genotype of both *CD14* -260 and *IL-6* -174. As no significant interaction could be found between the *CD14* -260 and *IL-6* -174

polymorphisms in the multivariate model, it could be assumed that the severe periodontal disease in these subjects reflects the load of the two separate SNPs. As CD14 and TLR4 together recognize the LPS-LPB complex, it was reasonable to study the possible interaction of these two polymorphisms as well. No such interaction could, however, be found. The fact that subjects with a positive genotype of *CD14* -260 or *IL-6* -174 and their combination generally had more sites with periodontal disease, but a similar amount of local etiology, when compared with the rest of the subjects, supports the hypothesis that a positive genotype predisposes to periodontitis.

Concerning TNF- α , several functional promoter variants have been described and the influence of the polymorphisms seem to be highly complex, with several variants having possible co-operative effects (Smith & Humphries 2009). Although we found a significantly higher extent of periodontal pocketing in subjects carrying the GG genotype than in those carrying the AA/AG genotype, a recent meta-analysis indicated a lack of association between the *TNF- α* -308 G/A polymorphism and susceptibility to chronic periodontitis (Nikolopoulos *et al.* 2008).

The findings of the above-mentioned meta-analysis also revealed a moderate positive association between the *IL-1A* -889 T variant and chronic periodontal disease in Caucasians. In addition, the report documented an elevated risk for chronic periodontitis in *IL-1B* +3954 T allele carriers, particularly in Asian-descents subjects (Nikolopoulos *et al.* 2008). Contradictory to the previous we did not find associations between single nucleotide polymorphisms in *IL-1A* -889 or *IL-1B* +3954 and periodontal disease. Nor could we confirm an association between *IL-10* -1082 and *TLR-4* +896 and periodontal disease reported elsewhere (Yoshie *et al.* 2007, Taylor *et al.* 2004).

One reason for the conflicting results concerning gene polymorphism in previous studies may be population heterogeneity, which is a problem in any population-based study of complex diseases discovering genetic effects. Disease prevalence often changes with geography and ethnic origin, and allele frequencies can vary widely throughout the world. Additionally, there is likely to be variation between populations of different origins in linkage disequilibrium (LD) mapping (Burgner *et al.* 2006, Palmer & Cardon 2005). Some of the studied alleles may not be associated with the disease themselves; instead, they may be in LD with the disease-associated allele. Both association studies and linkage analysis leave open the question of whether a disease-associated genetic variant is functionally important or serving only as a genetic marker with a functional locus co-inherited

on the polymorphic allele (Fife *et al.* 2005). If so, because LD breaks down differently in different populations, variations in the statistical analyses may reflect variable LD rather than the true genetic effect. In other words, a different allele of a single SNP may be a marker for certain phenotype in different populations. This may explain discordant results obtained for instance with *IL-6* -174 SNP in periodontitis.

Genes can interact with each other (epistasis) or with their environment (Burgner *et al.* 2006). Gene-environment interaction has been suggested to exist for instance between *CD14* -260 C/T polymorphism and *Helicobacter pylori* infection affecting serum concentration of immunoglobulin E (IgE). The authors hypothesized that the same genotype may increase, decrease or have no effect on total IgE depending on environmental factors, such as microbes (Virta *et al.* 2008). Polymorphisms of genes coding pro-inflammatory cytokines in periodontitis patients have been reported to be associated with the carriage of periodontal pathogens, such as *A.actinomycetemcomitans* and *P.gingivalis* (Nibali *et al.* 2007b, Laine *et al.* 2005). Lack of microbial data from our subjects prevents us from analyzing gene environment interactions in the way reported previously (Virta *et al.* 2008). Concerning the *IL-6* polymorphism and the genotype involved (G or C -containing), it has been suggested that the *IL-6* -174 effect may be dependent on interactions with other factors, such as the age of subjects. Recently, in the Cardiovascular Risk in Young Finns Study group of 2,228 subjects aged 24–39 years, *IL-6* -174 allele G associated with beneficial profile of early predictors of atherosclerosis (Hulkkonen *et al.* 2009). The authors suggested that in a sample of young adults there are less confounding factors, such as age-related chronic inflammatory processes, which are able to mask the effects of a single locus polymorphism.

6.3 Serum levels of IL-6 and sCD 14

In this study, periodontal infection was found to be a significant independent determinant for serum IL-6 and sCD14 concentrations in a dose-dependent way, and therefore, in spite of the local character of periodontal disease, it may be equated with systemic inflammatory and infectious diseases.

IL-6 plasma levels have been shown to correlate with the clinical manifestations of other diseases, such as coronary heart disease, in which IL-6 serum concentration may be a predictor of future cardiovascular events (Luc *et al.* 2003, Pradhan *et al.* 2002). In patients with primary Sjögren's syndrome IL-6

levels increased in parallel with increasing lesion grade in minory salivary gland biopsies and the number of primary Sjögren's syndrome criteria fulfilled (Hulkkonen *et al.* 2001). Elevated levels of IL-6 have also been reported to exist in serum and in the synovial fluid of affected joints in patients with rheumatoid arthritis (Cronstein 2007). Our results are in line with earlier studies suggesting that periodontal disease contributes to the systemic pool of circulating IL-6 (Loos 2005, Loos *et al.* 2000). One previous study (Ioannidou *et al.* 2006) also reported that the level of the clinical periodontal attachment was a significant independent predictor for elevated serum IL-6 levels within a heart and kidney transplant patient group. In that study serum IL-6 concentration was also positively associated with the level synthesized locally in the periodontal tissues. As regards serum sCD14, increased concentrations have been found not only in patients with periodontitis (Hayashi *et al.* 1999), but also in diseases such as asthma and rheumatoid arthritis (Yu *et al.* 1998, Garty *et al.* 2000, Martin *et al.* 2006).

The finding that the extent of periodontitis was found to be a significant independent determinant for serum IL-6 and sCD14 levels in a dose-dependent way was interpreted to mean that the low-grade systemic inflammation observed in these subjects was evoked by periodontal infection. The increase in serum IL-6 and sCD14 may be explained both by leakage of the locally produced IL-6 and sCD14 from the inflamed periodontal area and by systemic production in response to the systemically dispersed antigen load. Evidently, with an increasing systemic LPS or other bacterial product challenge, higher amounts of IL-6 and sCD14 are produced, and locally more of them are released into the circulation. sCD14 is produced by hepatocytes as a response to bacterial antigens or IL-6 stimulation (Bas *et al.* 2004). IL-6, on the other hand, is produced by various types of lymphoid and non-lymphoid cells, such as T cells, B cells, monocytes, fibroblasts and endothelial cells (Kishimoto 1989). Adipocytes are also known to produce IL-6 (Kershaw & Flier 2004). In fact, serum IL-6 levels were higher in subjects with higher BMI, and BMI turned out to be significantly associated with serum IL-6 concentration in the present subjects. Besides periodontal infection, the subjects reported to be systemically healthy. We cannot, however, fully exclude the possibility that some of them had sub-clinical medical conditions that may have affected their IL-6 or sCD14 serum levels.

In the present study *IL-6* -174 genotype was significantly associated with serum IL-6 level, and subjects carrying the GG genotype had higher serum IL-6 concentrations. Previously the G allele at position -174 in the promoter region has been linked to increased serum concentration of IL-6 in diseases such as juvenile

chronic arthritis (Fishman *et al.* 1998) and Sjögren's syndrome (Hulkkonen *et al.* 2001). Evidently the *IL-6* -174 genotype was not only an independent determinant for the increased serum IL-6 concentration in this study, but it also modified the association between serum IL-6 concentration and the extent of periodontal disease. There is only one previous study in which serum IL-6 concentration has been related to *IL-6* -174 genotype in periodontitis patients (D'Aiuto *et al.* 2004c). Contradictory to our results, however, higher serum IL-6 concentrations were reported in subjects with the CG/CC genotype than in those with the GG genotype. Another finding of that study was that also the levels of serum CRP were higher in subjects carrying the CG/CC genotype (D'Aiuto *et al.* 2004c). IL-6 is known to be among the cytokines that act on hepatocytes to induce acute-phase reactants including CRP (Gabay 2006). Although no CRP data were included here, the results of this study are in line with the previous, assuming that the *IL-6* -174 genotype is associated with systemic inflammation in periodontitis.

Recently new polymorphic sites have been described in both *IL-6* and *CD14* genes. There appear to be other polymorphic sites, at least -572, -1363, -1480, and -6106 of the *IL-6* gene (Fife *et al.* 2005, Terry *et al.* 2000, Nibali *et al.* 2008) and -1619 and -1359 –variants of the *CD14* gene (Le Van *et al.* 2008). The conflicting results of *IL-6* -174 allele G promoter activity might be explained by synergistic effects of the different single nucleotide polymorphisms (SNPs) and their function in linkage disequilibrium (Fife *et al.* 2005, Nibali *et al.* 2008). A recent study, the largest one to date, carried out a meta-analysis of the *IL-6* -174 polymorphism and serum IL-6 levels from 5,659 subjects (Huth *et al.* 2008). No association was seen in this sample between *IL-6* -174 polymorphism and circulating IL-6 levels, which adds further evidence to the existence of additional loci that may have an effect on IL-6 expression. However, the specific SNP at -174 position and its GG genotype seems to be a good marker for genetically determined hyperproduction of IL-6 in Finnish population, as shown in studies related to a systemic inflammatory disease (Hulkkonen *et al.* 2001).

After stratification by periodontal health status, the differences in serum sCD14 concentrations between genotypes were statistically significant only in the control group. Analogously to the previous, the *CD14* -260 polymorphism alone might not be sufficient to modulate serum sCD14 concentration; rather, sCD14 concentration is regulated by a combination of several SNPs (Le Van *et al.* 2008, Le Van *et al.* 2006). The reason for the lack of significant differences in the periodontitis group could also be that in the case of moderate to severe disease,

the infection may have had a dominant influence on the sCD14 level, overshadowing the significance of the genotype.

6.4 IL-6 genotype and periodontal disease in type 1 diabetics

In type 1 diabetic subjects *IL-6* -174 genotype was significantly associated with the extent of periodontal disease, those with the GG genotype exhibiting a significantly higher extent of bleeding on probing and periodontal pocketing than those carrying the CG/CC genotype. This result is basically consistent with our findings in the group of periodontitis patients without diabetes.

The glycemic control of type 1 diabetes over the preceding 3 years associated with the extent of periodontal disease; a finding which is in line with earlier studies (Lim *et al.* 2007, Mealey *et al.* 2006, Tervonen & Karjalainen 1997). One mechanism through which chronic hyperglycemia contributes to the severity of periodontal disease is that it accelerates the formation of the so-called advanced glycation end products (AGEs). AGEs in turn affect the secretion of cytokines and other inflammatory mediators through interacting by monocytic cell-surface receptors (RAGEs). In the context of the present study, one may hypothesize that hyperglycemia contributed to the monocytic release of IL-6 of the present type 1 diabetic subjects as well: the poorer the control of diabetes, the greater the production of both local and systemic IL-6 and the worse the periodontal health. The higher extent of periodontal disease in the GG genotype subjects, on the other hand, can be seen as a result of the previously shown higher production of IL-6 by subjects carrying this special genotype (Fishman *et al.* 1998, Hulkkonen *et al.* 2001). That a significant association between the extent of periodontal disease and the glycemic control could be found in subjects carrying the CC/CG, but not the GG genotype, may indicate that stronger factors, evidently the genotype itself, overshadowed the influence of glycemic control on periodontal disease in subjects carrying the GG genotype.

Several studies have investigated the role of cytokine gene polymorphisms in order to determine possible contributions to diabetes mellitus susceptibility. Concerning type 2 diabetes, in a joint analysis of individual-level data from 9440 study participants with type 2 diabetes or controls, C-allele carriers of *IL-6* -174 G/C polymorphism had significantly lower fasting glucose levels independently of BMI. These results indicate decreased risk of C-allele carrying subjects for type 2 diabetes. On the other hand, there was no evidence of an association between *IL-6* -174 G/C polymorphism and circulating IL-6 levels (Huth *et al.*

2009). Only marginal or inconsistent evidence has been published to support the role of the cytokine polymorphisms in the pathogenesis of type 1 DM (Cooper *et al.* 2007, Kristiansen & Mandrup-Poulsen 2005, Kumar *et al.* 2007). In a recent meta-analysis, however, the role of *IL-6* gene could not be excluded (Cooper *et al.* 2007).

In our study, the distributions of the subjects by the *IL-6* -174 genotype were similar among the diabetic and the reference subjects and thus no overrepresentation of either of the genotypes (GG and CG/CC) in the diabetic group could be observed.

7 Summary and conclusions

In this study, the associations between the extent of periodontitis and the single nucleotide polymorphisms in *CD14* -260, *IL-6* -174, *TNF- α* -308, *IL-10* -1082, *IL-1A* -889, *IL-1B* +3954 and *TLR4* +896 were investigated. No statistically significant differences were found in the frequencies of the cytokine genotypes between the periodontitis and the reference subjects when any of the single nucleotide polymorphisms were considered. However, carriage of the T-containing genotype of the *CD14* -260 and the GG genotype of the *IL-6* -174 were significantly associated with the extent of moderate to severe chronic periodontitis, indicating a role of genetic factors in the pathogenesis of the disease.

Both the extent of periodontal infection and the *IL-6* -174 genotype turned out to be significant determinants of the serum IL-6 levels. Subjects carrying the GG genotype had higher serum IL-6 levels than those carrying the CG/CC genotype. The serum level of sCD14 was significantly higher in subjects carrying the T-containing than the CC genotype of the *CD14* -260 in the control group, but not in the periodontitis group, suggesting that moderate to severe periodontal infection overshadows the influence of the genotype on serum sCD14 level.

The *IL-6* -174 genotype associated significantly with the extent of periodontal disease also in subjects with type 1 diabetes mellitus, the subjects with the GG genotype having more severe disease. A significant association between the glycemic control of diabetes and the extent of periodontal disease was observed in subjects carrying the GC/CC genotype, but not in those with the GG genotype. Thus, as a new finding we report that not only glycemic control but also the *IL-6* -174 genotype should be taken into consideration when analyzing the risk of periodontal disease in type 1 diabetic subjects.

In this study we found consistently significant associations between periodontal disease expression, serum levels of inflammatory markers, and genetic factors in a cross-sectional study design and in fairly small study samples. This comprehensive approach has not been used before, and the results obtained can be interpreted as suggestive of a causal relationship between the studied variables.

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Original articles

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

- I Tervonen T, Raunio T, Knuutila M & Karttunen R (2007) Polymorphisms in the CD14 and IL-6 genes associated with periodontal disease. *J Clin Periodontol* 34: 377–383.
- II Raunio T, Nixdorf M, Knuutila M, Karttunen R, Vainio O & Tervonen T (2007) The extent of periodontal disease and IL-6⁻¹⁷⁴ genotype as determinants of serum IL-6 level. *J Clin Periodontol* 34: 1025–1030.
- III Raunio T, Karttunen R, Knuutila M, Vainio O & Tervonen T (2009) Serum sCD14, polymorphism of CD14⁻²⁶⁰ and periodontal infection. *Oral Dis* 15: 484–489.
- IV Raunio T, Knuutila M, Hiltunen L, Karttunen R, Vainio O & Tervonen T (2009) The IL6⁻¹⁷⁴ genotype associated with the extent of periodontal disease in type 1 diabetic subjects. *J Clin Periodontol* 36: 11–17.

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Original publications are not included in the electronic version of the dissertation.

1012. Pajala, Ari (2009) Achilles tendon rupture. Comparison of two surgical techniques, evaluation of outcomes after complications and biochemical and histological analyses of collagen type I and III and tenascin-C expression in the Achilles tendon
1013. Tetri, Sami (2009) Factors affecting outcome after primary intracerebral hemorrhage
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