

**Department of Dental Medicine
Karolinska Institutet, Stockholm, Sweden**

**Institute of Dentistry,
University of Helsinki, Helsinki, Finland**

ASSOCIATIONS BETWEEN ORAL BIOFILM, PERIODONTAL DISEASE, AND SYSTEMIC HEALTH

**WITH A FOCUS ON ATHEROSCLEROSIS
AND BREAST CANCER**

Maha Yakob



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“He is happiest, be he king or peasant, who finds peace in his home.”

-Johann Wolfgang von Goethe (1749-1832)

To the love of my life; my family

SUPERVISORS:

Professor em. Birgitta Söder, Karolinska Institutet, Department of Dental Medicine

Professor Jukka H. Meurman, University of Helsinki, Institute of Dentistry

FACULTY OPPONENT:

Professor Bruno G. Loos, University of Amsterdam, Academic Center for Dentistry

EXAMINATION COMMITTEE:

Professor Maud Wikström, University of Gothenburg, Department of Odontology

Professor Anders Gustafsson, Karolinska Institutet, Department of Dental Medicine

Docent Pirkko Pussinen, University of Helsinki, Institute for Dentistry

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ABSTRACT

The general hypothesis of this series of studies was that oral infections, particularly periodontal disease, by triggering inflammatory reactions detrimentally affect systemic health where inflammations are known to play a role in the pathogenesis, namely cardiovascular disease and cancer. Consequently, the general aim was to study the association between oral biofilm and certain oral micro-organisms, periodontal disease, and selected inflammatory markers with a focus on atherosclerosis and breast cancer (BC).

In *Study I*, the aim was to examine the involvement of a high amount of dental plaque, severe gingival inflammation and periodontal disease in the development of early atherosclerotic lesions in women. The carotid arteries were examined with ultrasonography. Periodontal disease appeared to be a principal independent predictor in the development of atherosclerotic process women with periodontal disease. Our findings indicated that a high amount of dental plaque, severe gingival inflammation as well as periodontal disease seemed to be associated with the development of atherosclerotic lesions in women already at the early subclinical stage. In *Study III*, the aim was to examine early atherosclerotic changes in carotid arteries and relate the findings to the serum levels of high-sensitivity C-reactive protein (hsCRP) in subjects whose periodontal status have been followed for at least 18 years. Women had significantly lower hsCRP values and significantly higher high-density lipoprotein (HDL) cholesterol values than men. Nevertheless, women with periodontal disease had significantly more atherosclerotic lesions than women without periodontal disease. Increased levels of hsCRP could not discriminate the patient group from the control group for either men or women. Periodontal disease was identified as the major independent predictor of increased carotid artery lesions. Hence, periodontal disease might nevertheless present a risk for atherosclerotic disease, particularly in women, irrespective of low hsCRP levels.

In *Study II*, the aim was to evaluate the incidence of BC in subjects with periodontal disease and the characteristic tooth loss in a 16-year prospective investigation. Participants diagnosed with periodontal disease and BC had significantly more missing molars when compared with subjects with periodontal disease but without BC. The difference in the prevalence of BC in subjects with periodontal disease and with or

without any missing molar in the mandible was significant. Thus, chronic periodontal disease indicated by missing molars seemed to be associated with the incidence of BC.

In *Study IV*, the aim was to investigate in subjects with and without periodontal disease the levels of salivary albumin, total protein, and matrix metalloproteinases-8 (MMP-8), with or without the simultaneous presence of specific periodontal micro-organisms detected by polymerase chain reaction (PCR) in gingival crevicular fluid (GCF). The presence of both *Treponema denticola* and *Tannerella forsythia* associated with increased MMP-8 concentration in GCF. Furthermore, the presence of *T. denticola* associated with increased albumin and total protein concentrations in saliva. In *Study V*, the aim was to assess the association between site-specific subgingival micro-organisms and the levels of MMP-8 and MMP-9 at test sites. *T. denticola* was significantly more present at test sites in patients compared with the control group. Furthermore, the site -specific presence *T. denticola* in GCF appeared to increase the release of MMP-8 and MMP-9 at test sites. Thus, the results from *Studies IV* and *V* confirmed the assumption that periodontal micro-organisms might indeed trigger an inflammatory host-response.

Summing up, periodontal disease was found to be associated with subclinical atherosclerotic lesions and also a higher incidence of BC. Furthermore, *T. denticola* associated with increased salivary albumin, total protein as well as with higher levels of MMP-8 and MMP-9 in GCF, indicating a possible inflammation triggering capacity of the oral biofilm. Thus, our findings did confirm our primary hypotheses. The associations indicate that periodontal disease might pose a threat to systemic health.

Keywords: periodontal disease, periodontitis, oral biofilm, dental plaque, inflammation, atherosclerosis, breast cancer, gingival crevicular fluid, matrix metalloproteinase, C-reactive protein, carotid plaque, micro-organisms, salivary proteins, *Treponema denticola*

LIST OF PUBLICATIONS

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

- I. Söder B, **Yakob M**, Nowak J, Jogestrand T. Risk for the development of atherosclerosis in women with a high amount of dental plaque and severe gingival inflammation. *International Journal of Dental Hygiene* 2007 Aug;5(3):133-8.
- II. Söder B, **Yakob M**, Meurman JH, Andersson LC, Klinge B, Söder PÖ. Periodontal disease may associate with breast cancer. *Breast Cancer Research and Treatment* 2011 Jun;127(2):497-502.
- III. **Yakob M**, Meurman JH, Jogestrand T, Nowak J, Söder PÖ, Söder B. C-reactive protein in relation to early atherosclerosis and periodontitis. *Clinical Oral Investigations* 2010 Dec 7. [Epub ahead of print].
- IV. **Yakob M**, Kari K, Tervahartiala T, Sorsa T, Söder P-Ö, Meurman JH, Söder B. Associations of periodontal micro-organisms with salivary proteins and MMP-8 in gingival crevicular fluid. *Journal of Clinical Periodontology* 2011 Nov 21. [Epub ahead of print].
- V. **Yakob M**, Meurman JH, Sorsa T, Söder B. Site-specific presence of *Treponema denticola* associates with increased levels of MMP-8 and MMP-9 in gingival crevicular fluid. *Submitted*.

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LIST OF ABBREVIATIONS

<i>A.a.</i>	<i>Aggregatibacter actinomycetemcomitans</i> (<i>A. actinomycetemcomitans</i>)
ANOVA	Analysis of variance
BC	Breast cancer
BH	Bone height
BMI	Body mass index
BOP	Bleeding on probing
CAL	Clinical attachment loss
CI	Calculus index or Confidence interval
cIMA	Calculated intima-media area
CRP	C-reactive protein
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
CVD	Cardiovascular disease
GCF	Gingival crevicular fluid
GI	Gingival index
HDL	High-density lipoprotein
hsCRP	High-sensitivity C-reactive protein
ICD	International classification of diseases
IFMA	Immunofluorometric assay
IgA	Immunoglobuline A
IgG	Immunoglobuline G
IgM	Immunoglobuline M
IMT	Intima-media thickness
LD	Lumen diameter
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MI	Myocardial infarction
MMP	Matrix metalloproteinase
OR	Odds ratio
PCR	Polymerase chain reaction
PD	Pocket depth
<i>P.g.</i>	<i>Porphyromonas gingivalis</i> (<i>P. gingivalis</i>)
<i>P.i.</i>	<i>Prevotella intermedia</i> (<i>P. intermedia</i>)
PLI	Plaque index
PMN	Polymorphonuclear
SD	Standard deviation
<i>T.d.</i>	<i>Treponema denticola</i> (<i>T. denticola</i>)
<i>T.f.</i>	<i>Tannerella forsythia</i> (<i>T. forsythia</i>)
TG	Triglycerides
TIMP	Tissue inhibitor of metalloproteinases
WHO	World health organization

INTRODUCTION

ORAL BIOFILM

Van Leeuwenhoek, a scientist from the Netherlands, commonly known as “the Father of Microbiology”, was the first to observe and describe single celled organisms, which we now refer to as micro-organisms. The oral micro-organisms were the first to be studied by Van Leeuwenhoek, already in the year 1683, by using a handcrafted microscope (1). Oral biofilm plays a key role in the aetiology of oral disease. Biofilms are microbial communities composed of numerous diverse organisms that exist in a collective state (2, 3). Dental plaque has been defined as a microbial community that develops on the tooth surface, embedded in a matrix of polymers of microbial and salivary origin. Dental plaque is a microbial community with a very complex structure and marked genetic diversity (4).

The oral microbial community is one of the most intricate microbiota in the human body. Both pathogenic and commensal micro-organisms co-exist in the oral biofilm (5). The composition of the micro-organisms differs between the dental plaque above the gingival margin (supragingival plaque) and that below the gingival margin (subgingival plaque) (2, 6, 7). A pellicle consisting of proteins and glycoproteins is formed promptly after cleaning of the tooth surface, and the supragingival plaque starts to develop (8). Since a small number of micro-organisms can multiply fast, the re-colonization of micro-organisms on oral surfaces after physical removal occurs rapidly and consists mostly of Gram-positive, facultative cocci, *Streptococcus* and *Actinomyces* species (9).

Experimental research and clinical trials have repeatedly confirmed the importance of effective removal of dental plaque to maintain oral health (10-12). The role of dental plaque was reported to be associated with oral inflammatory diseases more than half a century ago (13-15). Effective removal includes good oral hygiene together with interproximal cleaning, in addition to regular biofilm removal by professionals (16). Bacterial enzymes in the dental plaque can directly and indirectly, as a consequence of the host inflammatory response, cause destruction of tissues and lead to periodontal diseases (17).

PERIODONTAL DISEASES

Periodontal diseases involves the supporting tissues of the teeth and generally refers to the inflammation of the gums (gingivitis) and the tissues surrounding the teeth (periodontitis) (18). Gingivitis, a common inflammation in the periodontal tissue, is caused by accumulation of dental plaque. Dental plaque develops on the teeth within 24 hours, causing gingivitis in 10-21 days (10). Some of the early clinical signs of gingival inflammation are redness of the gingival margin, swelling and spontaneous bleeding. Both patients and dental practitioners underestimate the disease though, since chronic gingivitis seldom manifests with spontaneously bleeding and is often painless (19).

Periodontitis is characterized by inflammation that extends deep into the tissue, destruction of supporting connective tissue, and alveolar bone loss. One clinical indication of periodontitis is the developing of soft tissue pockets between the gingiva and the tooth root (**Figure 1**). Even though chronic periodontitis is usually asymptomatic, it can result in loosening of teeth, sporadic pain and discomfort, ultimately leading to tooth loss (20-22). Furthermore, it seems that gingivitis precedes periodontitis, teeth that consistently are surrounded by inflamed gingival more frequently lead to tooth loss (19).

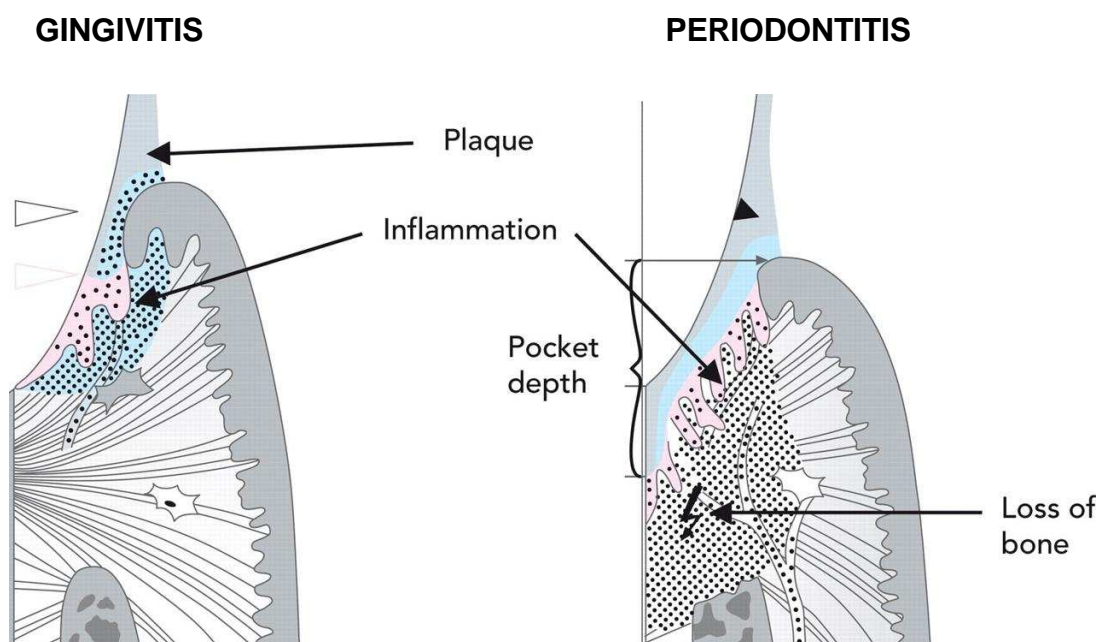


Figure 1. Gingivitis is an inflammatory condition affecting the supra alveolar periodontal tissues, while periodontitis is a chronic inflammatory condition affecting deeper periodontal tissues, such as the connective tissue attachment and bone. Reprinted with permission from Oxford University Press, from Sanz *et al.* (2010) (23).

Mild to moderate periodontitis affects as many as 20-50% of adults, and severe, generalized periodontitis is seen in about 5-20% of adults (24, 25). Nevertheless, any information about the prevalence must take into account the study population as well as the definition of periodontitis used (25, 26).

Periodontitis is a multi-factorial disease with a complex pathogenesis (18). Most of the tissue destruction occurs as a consequence of the host immune-inflammatory response, although micro-organisms are the main etiological agents. Furthermore, the response is altered by both genetic and environmental risk factors (27). Smoking is one of the most recognized risk factors for periodontitis. Smokers are reported to be much more susceptible to developing periodontitis than non-smokers (28, 29). Increased age, genetic predisposition, diabetes, psychosocial stress and several uncommon systemic diseases are also considered risk factors for periodontitis (20, 26, 27).

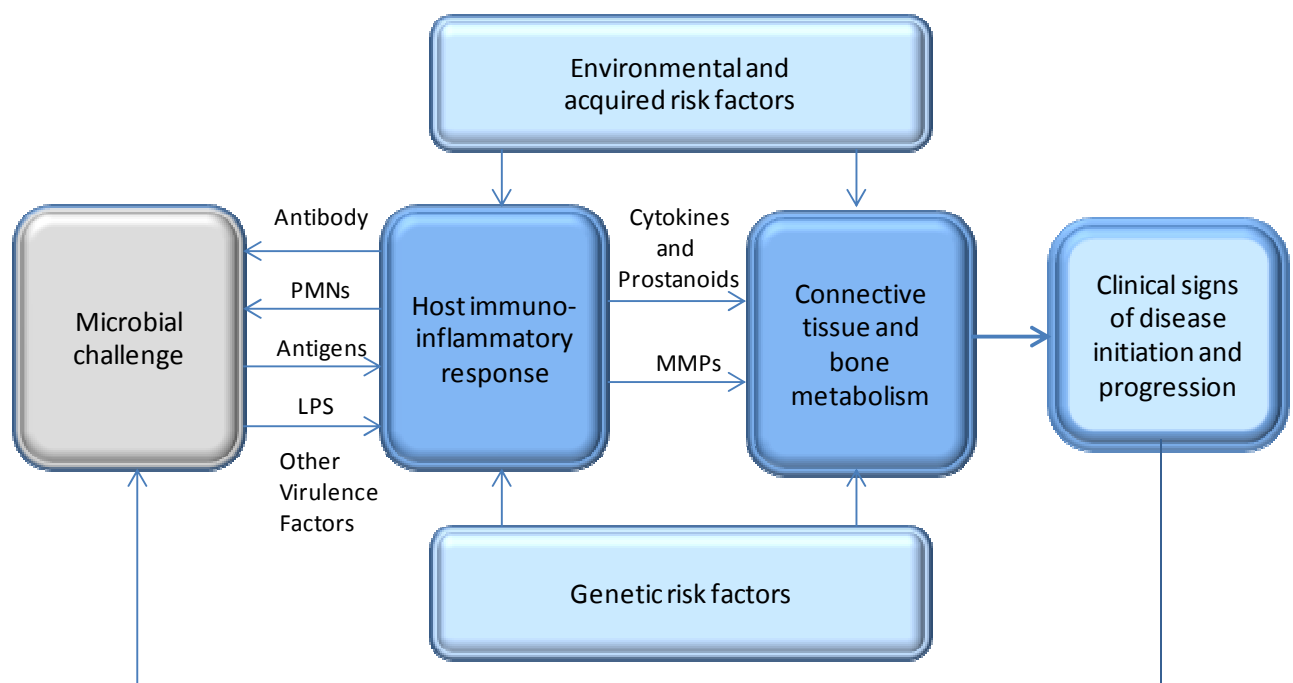


Figure 2. Model of pathogenesis of periodontitis adapted from Page and Kornman (1997). Periodontitis is a complex disease, and although micro-organisms are the main etiological agents, most of the tissue destruction occurs as a consequence of the host immune-inflammatory response. Furthermore, both genetic and environmental risk factors (e.g. smoking) can affect the response (27). (PMNs: polymorphonuclears, LPS: lipopolysaccharide, MMPs: matrix metalloproteinases)

CLINICAL CONCEPTS AND THERAPY

The clinical diagnosis of periodontitis is usually based on both visual and radiographic assessment. Destruction of the periodontal tissue is measured by estimating the spaces between tooth enamel and cement and gingiva (30), which are normally 1-3 mm (31). Pocket depths, the amount of dental plaque, calculus, and gingival bleeding are registered during a clinical examination to diagnose existing disease, to determine the prognosis, and to monitor disease progression (30).

The basis for periodontal therapy is to mainly control the biofilm with anti-infective non-surgical treatment. Disease progression can be inhibited for many years by controlling the periodontal biofilm with professionally administered oral hygiene (32). The goal with disrupting the biofilm is to decrease the microbial load, leading to a decrease in substances associated with periodontitis (33). The most effective therapy to eradicate periodontal infection and control gingival inflammation is by combining supra- and subgingival debridement with oral hygiene instructions (34). A continual absence of gingival inflammation is necessary to achieve a successful periodontal therapy (11).

If periodontal infection remains after the healing phase, periodontal surgery could be required to remove subgingival microbial deposits, combined with tissue regeneration procedures to restore parts of the lost periodontal tissues (34). In cases where patients do not respond well to the mechanical therapy or in cases with a more aggressive disease form, the use of systemic antibiotics in combination with mechanical periodontal therapy is recommended (35). Novel treatment approaches have focused on developing drugs that suppress host-destructive pathways, e.g. subantimicrobial doses of doxycycline have anticollagenolytic functions and suppress tissue destruction (36).

Gingivitis is preventable and reversible with daily oral hygiene (10). Successful treatment might eliminate inflammation and may even regenerate some bone and connective tissue, but complete restoration of the lost tooth support is impossible and periodontitis is therefore considered an irreversible disease (20).

PERIODONTAL MICROBIAL ECOLOGY

The human oral cavity contains numerous different habitats colonized by micro-organisms. More than 700 of bacterial species have been identified in the human oral cavity and most of them in dental plaque (37-39). The structure and composition of supragingival flora differs strikingly from those of subgingival flora (40). The total number of microbial cells in subgingival plaque is estimated to be 33×10^8 in healthy subjects, compared with 174×10^8 in periodontitis patients (2).

Porphyromonas gingivalis and *Tannerella forsythia* are Gram-negative bacteria associated with bone loss and together with the spirochete *Treponema denticola* they form the “red complex”, a cluster that is detected more frequently in subjects with periodontitis (39, 41, 42). The presence of the “red complex” species in subgingival plaque is associated strongly with pocket depth and bleeding on probing. Sites harboring all three species of the “red complex” revealed the deepest mean pocket depth (17). Subgingival counts are higher in subjects with periodontitis than in the periodontally healthy, and the major differences are indeed found primarily among the species of the “red complex” (2). *Aggregatibacter actinomycetemcomitans* is a Gram-negative facultative anaerobe (43) found more frequently in subjects with periodontitis and commonly present in juvenile periodontitis (44). *Prevotella intermedia* belongs to the “orange complex” and is also one a predominant species in the gingival crevice of subjects with severe periodontitis (9, 17).

The healthy oral cavity harbours a characteristic microbial flora that differs from that of oral disease (37). Aas *et al.* (2005) examined subgingival plaque samples from nine sites from five clinically healthy subjects. The micro-organisms of the “red complex” were not detected at test sites by culture-independent molecular techniques (37). Even if accumulation of micro-organisms on teeth is essential to the initiation and progression of periodontitis, and the predominant microbiota found in disease differs from that in health, there is no evidence that a single or unique pathogen is causative (37, 45).

HOST-RESPONSE FACTORS

Periodontitis is initiated by a microbial infection, followed by a host-mediated destruction of soft tissue. Thus, tissue destruction and alveolar bone loss are the result of prolonged microbial challenge and degenerative inflammation (18, 33, 46-48). Inflammation is a biological response caused by pathological stimuli to protect the organism, remove harmful stimuli and to start the healing process of the tissue (49, 50). Inflammatory mediators originate from plasma proteins or cells, including mast cells, platelets, neutrophils, monocytes/macrophages, and B- and T-cell lymphocytes, and they play a significant role in the process of inflammation (33, 51, 52). Hyperactivated or primed leukocytes produce cytokines, eicosanoids, and metalloproteinases that cause destruction of connective tissue and bone (33).

The subgingival microflora can produce toxins that provoke an immune-inflammatory response, leading to host-mediated tissue destruction (53-55). Lipopolysaccharides (LPSs) and proteases are example of toxins that can trigger tissue destruction. LPS is a very potent toxin that can elicit several pathophysiological effects (33). LPS can trigger macrophages to produce cytokines, which play a key role in periodontal bone resorption (18, 56). Although micro-organisms can initiate periodontitis, the disease outcome depends on host-response factors and environmental and genetic factors (27, 46, 57) in agreement with the basic principles of infectious diseases. Studies of twins have shown that approximately 50% of the variations in the severity of periodontitis is explained by genetic influences (57, 58). Genetic polymorphisms have been coupled with periodontitis (59), but evidence in support of the use of genetic tests to assess risk or predict treatment response is still lacking (60-62).

Saliva

Saliva is mainly produced by the major glands (parotid, submandibular and sublingual) and by hundreds of minor salivary glands. Saliva is a complex oral fluid that consists of a mixture of secretions from all of the salivary glands (63), and can contain immunoglobulin's (Ig), cytokines and other glycoproteins (64).

Specific antibodies in saliva have been examined for their diagnostic potential (65, 66). Patients with periodontitis have been reported to have higher IgA and IgG levels in saliva than non-periodontitis control subjects (67). Saliva can also include non-salivary components such as gingival crevice, serum, and food deposits (68). A range of protein

components have been identified in saliva, but a detailed understanding of its protein composition has not yet been established (69). The concentrations of albumin in saliva reflect a passive contribution of serum-derived proteins, which could potentially be due to epithelial inflammation. Changes in albumin concentrations of saliva can be used as a marker for systemic diseases (70, 71). Diminutive amounts of albumin are normally detected in the saliva of orally healthy individuals, and the concentrations are significantly increased in the transition to gingivitis or periodontitis (72).

Gingival crevicular fluid (GCF)

Gingival crevicular fluid (GCF) is a filtrate of blood and an exudate of the inflamed periodontal tissue (73, 74), with a recognized diagnostic potential (75-78). GCF contains leukocytes, particularly polymorphonuclear (PMN) granulocytes, host-derived molecules from blood, and substances from micro-organisms of the dental plaque (79). The ecology of the gingival crevice is highly anaerobic and differs from that of other sites in the oral cavity (2, 80). GCF may indicate systemic disease since it contains most of the humoral and cellular defense factors found in serum. GCF also has a number of complex proteins and glycoproteins that can serve as novel substrates for microbial metabolism (75, 81-84). The collection and analysis of GCF provide a useful, non-invasive, site-specific method to assess and monitor the pathophysiological status of the periodontium (85-87). The flow of GCF is relatively slow at healthy sites, while its production rises during inflammation in periodontitis (88), from approximately 3 $\mu\text{L/h}$ in healthy sites and up to 44 $\mu\text{L/h}$ in periodontitis-affected sites (74). This present thesis focuses on the presence of certain subgingival micro-organisms in GCF, as well as the levels of selected host-derived enzyme in GCF.

Matrix metalloproteinase (MMP)

Matrix metalloproteinases (MMPs) are zinc-dependent proteases considered to play important roles in diseases such as arthritis, periodontitis, and atherosclerosis, and in cancer cell invasion and metastasis (89-91). They are involved in the process or degradation of various extracellular, pericellular and non-matrix substrates (91-95). Most of the MMP activity and expression is undetectably low or at quiescent levels in intact normal tissues (85, 95). The activity of the MMPs is regulated by their endogenous inhibitors, the tissue inhibitors of metalloproteinases, known as TIMPs. An imbalance between MMPs and TIMPs can contribute to a range of inflammatory and malignant pathological processes (96).

At present, 25 members of the MMPs multigene family have been recognized and categorized, 24 of which are found in the human genome (91-95, 97). MMP-8 (collagenase-2) is synthesized during maturation of the PMNs in the bone marrow. MMP-8 can also be produced by articular chondrocytes and by resident synovial and gingival fibroblasts, epithelial cells/keratinocytes, odontoblasts, oral cancer cells, monocytes/macrophages and plasma cells during different inflammatory diseases such as arthritis and periodontitis (98-103). MMP-9 (92 kDa gelatinase B) can be expressed by different cell lines such as keratinocytes, osteoclasts, eosinophils, neutrophils, and macrophages (91, 95, 103-105).

MMP-8 and MMP-9 derived from PMNs, have been reported to be the most common MMPs in periodontitis-affected GCF (95, 105). The intensity and degree of MMP-8 and MMP-9 activation have been reported to be enhanced with increasing periodontal disease severity, and to decrease after periodontal treatment (85, 86, 95, 106). The biological function of the association between periodontitis and MMP-8 levels has not yet been clarified, but elevated levels of MMP-8 could contribute to the excessive and sustained proteolysis that results from the destruction of periodontal tissues (107).

The plasma levels of MMP-8 and MMP-9 were increased in myocardial infarction (MI) patients (108). An increased level of MMP-8 expression has been reported to be a good biomarker for cardiovascular outcomes since it is correlated with atherosclerotic plaque vulnerability (109, 110). MMPs also play a role in malignant pathological processes (111). Even though MMP-8 plays a role in the development of an inflammatory response, a anti-inflammatory role during recovery has also been described (112). Increased MMP-8 expression in breast cancer seems to inhibit the ability of the cancer cells to spread and MMP-8 has been reported to have an antitumor function (113, 114).

PERIODONTITIS AND CARDIOVASCULAR DISEASE (CVD)

Cardiovascular diseases (CVDs) with outcomes such as MI, are common in adults and a leading cause of death (115). Elevated levels of low-density lipoprotein (LDL) cholesterol, hypertension, smoking, male gender, and low socioeconomic status are some of the risk factors for CVD (116). The Framingham Heart Study showed that low high-density lipoprotein (HDL) cholesterol was a stronger risk factor for CVD than high LDL cholesterol (116, 117).

The proposed theory that oral infections, particularly periodontitis, confer an independent risk for CVDs has been studied in several epidemiological investigations (118-123). An association between acute MI and periodontitis was first described by Mattila *et al.* (1989) (124), followed by studies reporting that individuals with periodontitis had an increased risk of subsequent CVD (125, 126). A meta-analysis concluded that periodontitis is an independent but weak risk factor for CVD, with a relative risk of 1.15-1.19 (127).

Plausible biological mechanisms for an association between periodontitis and atherosclerotic CVD have been proposed, but a direct causal relationship has yet to be established (23, 120). By measuring biomarkers in the plasma, periodontitis has been associated with increased systemic inflammation (128). LPS and other products from Gram-negative micro-organisms may be able to stimulate cytokine production, activate monocytes, and provoke release of acute phase proteins (51, 129). Furthermore, studies have shown that periodontitis and atherosclerosis are associated with endothelial dysfunction (119, 130). Endothelial dysfunction and evidence of systemic inflammation has been reported in subjects with severe periodontitis (131), which could be one potential mechanistic explanation for the link between periodontitis and CVD. Recently, Tonetti *et al.* (2007) conducted a randomized, clinical trial to evaluate the effect of periodontal therapy on endothelial function within six months. The authors reported that intensive treatment of periodontitis first resulted in acute, short-term systemic inflammation and endothelial dysfunction, but after the six-month observation period in improved endothelial (132). Some other studies have also shown an improvement in endothelial dysfunction after periodontal therapy (133). A state of endothelial dysfunction in the vascular wall could be the result of a repeated systemic exposure of oral micro-organisms, bacterial endotoxins, and systemic inflammation

(23, 134-136). However, the mechanism by which periodontitis affects endothelial function remains unknown.

Another mechanism in the link between periodontitis and CVD could be that the microbial pathogens derived from the subgingival biofilm are involved in the pathogenesis of atheroma plaque formation (**Figure 3**) (137, 138). Some oral bacteria species associated with the pathogenesis of periodontitis have further been detected in atheroma plaque (139-141). Viable oral bacteria could not be isolated from the atheromas, even when DNA of periodontal pathogens was detected in atherosclerotic plaques (142). Pussinen and co-workers uncovered an association between markers of *A. actinomycetemcomitans* and *P. gingivalis* infections in serum and future stroke (143) and increased risk of MI (144, 145). Clinical trials have reported temporary bacteremia after different dental procedures such as scaling, periodontal surgery, and dental extraction (146). However, oral bacteria are more frequently exposed to the vascular system by such normal daily actions as tooth brushing and chewing (147).

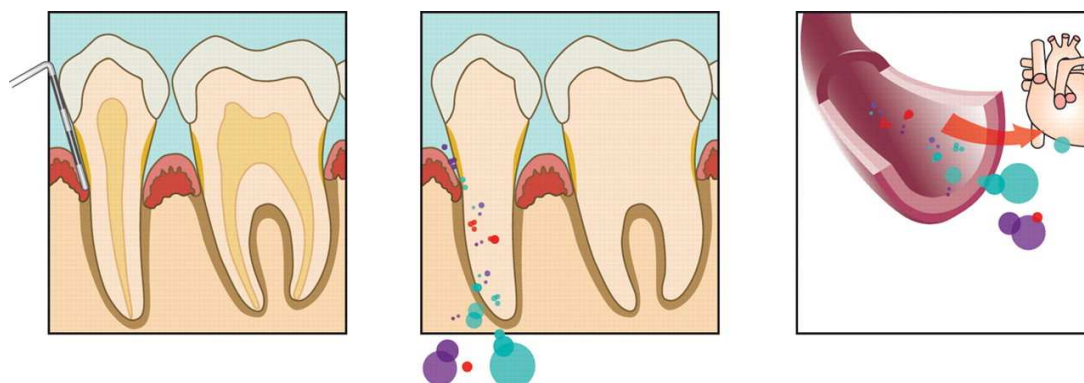


Figure 3. Potential model of pathogenesis of periodontitis and ischaemic cardiovascular diseases. A systemic dissemination of certain oral bacteria and their metabolites might have a direct or indirect influence on the pathophysiology of the atheroma. Reprinted with permission from Oxford University Press, Sanz *et al.* (2010) (23).

Many shared risk factors, such as diabetes mellitus, obesity, lipids, and hypertension, are common both in periodontitis and CVD and speak for an indirect relationship, confounders, such as smoking, have therefore to be considered in this regard (148, 149). Subjects with poorly controlled diabetes (both types 1 and 2) are more likely to have periodontitis than individuals without diabetes (150). Other confounders, including depression, physical inactivity, family history of CVD and periodontitis, advanced age, and male gender are important as well (120, 151, 152).

Atherosclerosis

Atherosclerosis is a low-grade chronic systemic inflammation that has been linked to adverse cardiovascular outcomes (153). One of the main risk factors for atherosclerosis is high plasma concentrations of cholesterol, particularly LDL. The process of atherogenesis is widely considered to consist of the accumulation of lipids within the artery wall (154). Lifestyle changes and use of novel pharmacologic approaches have been recommended to reduce plasma cholesterol concentrations (155). Still, atherosclerotic CVD is one of the major causes of death and approximately half of the patients with CVD do not have hypercholesterolemia. Thus, factors besides hypercholesterolemia might be of importance (153, 156, 157).

Inflammation has a key role in the pathogenesis of atherosclerosis; it is presumably involved in the initiation, but also in the progression to infarction (158). The cellular interactions in atherogenesis are similar to those of other chronic inflammatory diseases such as rheumatoid arthritis (159). Atherosclerotic lesions mainly affect large and medium-sized elastic and muscular arteries and might exist without complications. Plaque rupture or thrombosis can, however, lead to ischemia of the heart, brain, or other parts of the body and result in infarction (153). Smooth muscle cells proliferate and monocyte-derived macrophages and T-cells replicate during growth of atherosclerotic lesions. Monocytes are present in the process of atherogenesis, and monocyte-derived macrophages secrete cytokines, chemokines, growth-regulating molecules, MMPs and other hydrolytic enzymes (153).

Thickness of artery wall

Numerous clinical studies have used surrogate markers of subclinical atherosclerosis to verify the advantageous effects of different intervention on the progression of atherosclerosis (160-165). Measurement of carotid arterial wall thickness is mostly used since it is non-invasive and easy to access by high-resolution ultrasounds (160, 163). The intima-media wall thickness (IMT) of the carotid artery is a common measure of preclinical atherosclerosis, and increased IMT (≥ 1 mm) is associated with a higher risk for acute MI and stroke (166, 167). IMT can be affected by changes in the tissue mass in the inner layer as well as by simultaneous widening of the vessel. Increased diastolic blood pressure or a compensatory widening in atherosclerotic wall changes could thus decrease the IMT. By calculating intima-media area (cIMA) the potential source of error, can be overcome (163).

Associations with both periodontitis and tooth loss has been reported using IMT as a variable (168). The ARIC study observed that in patients with severe periodontitis there was a 30% increased risk of having IMT ≥ 1 mm, suggesting that subclinical atherosclerosis is present in patients with periodontitis (130, 169).

C-reactive protein (CRP)

Biomarkers with high predictive value for future cardiovascular events, mainly serum proteins involved in the pathophysiology of atherosclerosis, have been studied (170-177). The markers for cardiovascular risk are intended to be utilized in clinical trials and preventive programs (178). The most studied biomarker, C-reactive protein (CRP), has been considered a key marker of atherosclerosis and a risk predictor for CVD (179, 180). CRP, mainly produced by the liver, is an acute-phase protein that is normally present at low levels in plasma. In a response to inflammatory stimuli, the levels of plasma CRP can increase markedly (181, 182). Already in the 1980s, CRP was reported to be increased in patients admitted for acute MI (183).

An increased level of CRP in serum has been reported in subjects with periodontitis (184-188). Subjects with advanced periodontitis had higher serum levels of high-sensitivity CRP (hsCRP) than healthy controls (131). CRP levels seemed to be consistently higher (>2.1 mg/L) in patients with periodontitis than in healthy or gingivitis controls according to a recently published systematic review (128). Furthermore, a reduction in CRP after intensive periodontal therapy has been reported (189-191).

PERIODONTITIS AND CANCER

Carcinogenesis has been described as “a multistep process and these steps reflect genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives” (192). Several reports have shown that malignancies are initiated by infections (174, 176, 193, 194), and around 15% of the malignancies globally are caused by infections, comprising to 1.2 million cases each year (174). Viral infections caused by human papilloma virus (HPV) have been associated with oral cancer (195, 196). The oral cavity is an important potential source of both infection and inflammation, contributing to the total burden of disease and to overall health and well-being (197, 198). Oral infections are often asymptomatic but can nonetheless lead to bacteremia with no clear symptoms (199, 200). However, the mechanisms of the micro-organisms involved in carcinogenesis remain to be established (201, 202).

Good oral hygiene reduced the risk for esophageal carcinoma (203), and periodontal disease was linked with an higher risk of cancer in the pancreas among male health professionals. Furthermore, increased severity of periodontal disease, manifested as recent tooth loss, was associated with the greatest risk (204). Periodontal disease has also been related to tongue cancer (205) and head and neck cancer (206). Oral infections and inflammatory conditions, such as periodontitis, might indeed trigger malignant transformation in organs other than the mouth (**Figure 4**), but the scientific evidence is still weak and further studies are needed (177).

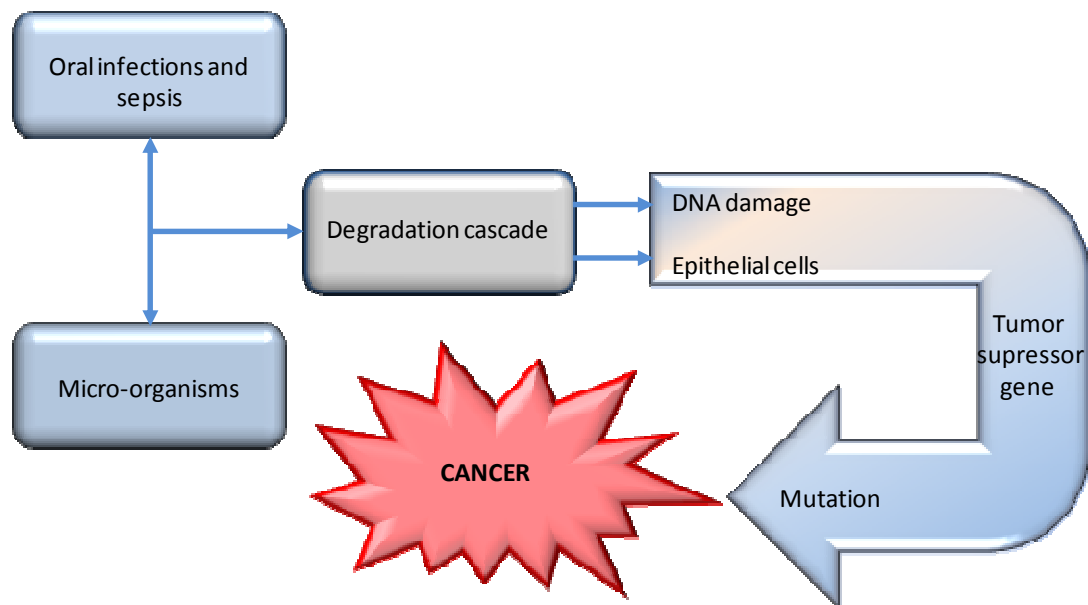


Figure 4. Potential pathway in the relationship between oral infections and cancer, adapted from Meurman and Bascones (2010) (177).

Breast cancer (BC)

Breast cancer (BC) is the most common malignancy and a leading cause of cancer mortality among women in most parts of the world (207, 208). An estimated 7.6 million deaths worldwide are caused by cancer and around 1 million women are diagnosed with BC each year. Of women diagnosed with BC, 40% die from the disease (209), and the vast majority of these deaths are due to metastasis (207, 208). In Sweden, BC represents 30% of all cancer cases in women and approximately 7000 cases are reported annually (210). Even though BC is a disease that mainly affects the elderly, 1 of 8 invasive cases are found in women under the age of 45 year (211). Prolonged exposure to endogenous estrogen, by early menstruation onset or delayed menopause, is strongly associated with BC. However, contradictory results have also been reported, and certain life changes such as increased exposure to toxins, imbalanced nutrition and a reduced amount of physical activity have to be considered as well (212, 213).

BC comprises numerous subtypes and is thus an extremely heterogeneous disease. Cancer originating in mammary epithelial cells, adenocarcinoma, is the most common histological type of BC, and this group contains 18 different subtypes according to the World Health Organization (WHO) (214).

By evaluating prognostic and predictive markers a treatment plan for BC is determined. A combination of the different therapy approaches, including surgery, chemotherapy and other drugs is commonly used (215, 216). The relative survival rate is higher nowadays due to early diagnosis, social awareness, and proper treatment (210).

AIMS OF THE THESIS

GENERAL HYPOTHESIS AND AIM

The general hypothesis of this series of studies was that oral infections, particularly periodontal disease, by triggering inflammatory reactions detrimentally affect systemic health where inflammations are known to play a role in the pathogenesis, namely CVD and cancer. Consequently, the general aim was to study the association between oral biofilm and certain oral micro-organisms, periodontal disease, and selected inflammatory markers with a focus on atherosclerosis and BC.

SPECIFIC AIMS

Study I examined the involvement of a high amount of dental plaque, severe gingival inflammation and periodontitis in the development of early atherosclerotic lesions in women.

Study II evaluated the incidence of BC in subjects with periodontal disease and characteristic tooth loss in a 16-year prospective investigation.

Study III assessed early atherosclerotic changes in carotid arteries and related the findings to hsCRP levels in subjects whose periodontal status have been followed for at least 18 years.

Study IV examined subjects with and without periodontitis for levels of salivary albumin, total protein, and MMP-8 in GCF, with or without the simultaneous presence of specific subgingival micro-organisms.

Study V evaluated the association between site-specific subgingival micro-organisms and levels of MMP-8 and MMP-9 in GCF.

MATERIALS AND METHODS

ETHICAL CONSIDERATIONS

The study protocol, methods and selection of subjects were approved by the Ethics Committee of Karolinska University Hospital, Karolinska Institutet, Huddinge, Sweden. All studies followed the principles for medical research according to the guidelines of the Declaration of Helsinki. The subjects received verbal as well as written information, and all subjects gave their informed consent to participate.

STUDY POPULATION

In 1985, a longitudinal prospective study was carried out with a random sample cohort of 3273 subjects. The subjects were randomly selected from a database registry of all inhabitants ($n= 105\,798$) of Stockholm county born on the 20th of any month from the year 1945 and 1954 (*Study II*). They were informed about the purpose of the study and offered a clinical oral examination. In total, 1676 subjects (51.2%; 838 women and 838 men) underwent a clinical oral examination in 1985. In 2001-2003, age- and gender-balanced subjects were selected from the cohort with a computer program to form two groups, those with periodontal disease ($n= 100$) and those without ($n= 50$) (*Studies I and III*). Between 2008 and 2009, a follow-up study of the same subjects was carried out (*Studies IV and V*). The clinical data originate from 1985, 2001-2003 and 2008-2009, and the registry data from 2001 (**Figure 5**). The subjects have also been described in earlier studies (217-222).

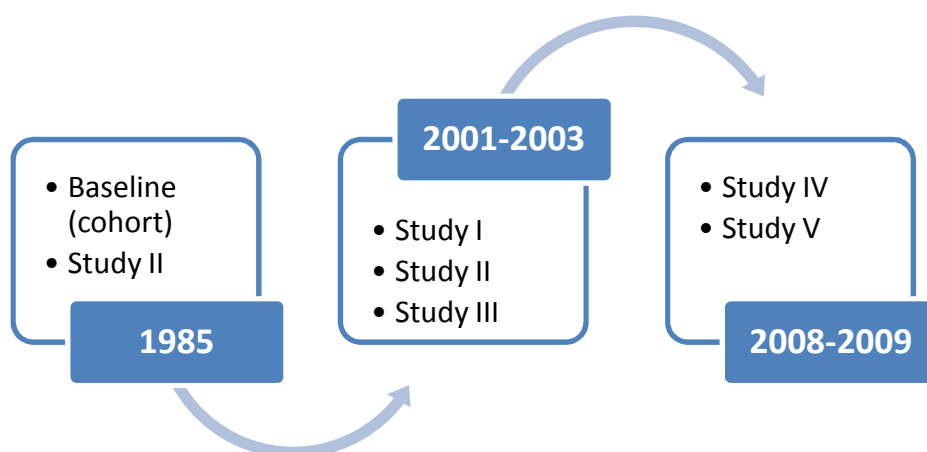


Figure 5. All subjects were randomly selected from a database registry in 1985. The figure provides an outline of when the data for the five studies were collected.

Regarding the drop-outs; 8 subjects were excluded in 2001-2003, and 8 did not participate. Thus, in total 134 subjects out of the 150 recruited subjects were examined in 2001-2003. In 2008-2009, the same subjects were recalled to participate in a clinical examination. The most given reasons for the drop-outs in 2008-2009 was lack of time ($n= 20$), few subjects were incapable due to illness ($n= 2$), and the rest could not be reached ($n= 11$). 101 subjects underwent a clinical examination, and 2 were excluded since they only had 2 teeth left, and consequently 99 subjects are analysed in *Studies IV* and *V*.

A flow-chart including all subjects and when the data collection was carried out, is presented in figure 6. *Study I* comprises 67 female subjects. Of these women, 46 women were randomly chosen from the group of individuals in whom the presence of periodontal disease was documented in 1985 and verified in 2001-2003 (patient group). At the same time, 21 women without periodontal disease were randomly selected as controls. In *Study II*, BC incidence among the baseline cohort of 3273 subjects until the year 2001 was analyzed. The clinically examined group in 1985 (Group A, $n= 1676$) and the remaining cohort group that were not examined in 1985 (Group B, $n= 1597$) were included in the analyses in *Study II*. In *Study III*, 134 subjects examined in 2001-2003 were included (67 women and 67 men), comprising subjects with and without periodontitis. The 67 women in this study are the same women described in *Study I*. *Studies IV* and *V* comprised 99 subjects. 53 subjects had periodontitis (24 women, 32 men) and were therefore classified as patients, while 46 subjects (22 women, 21 men) served as controls (**Figure 6**).

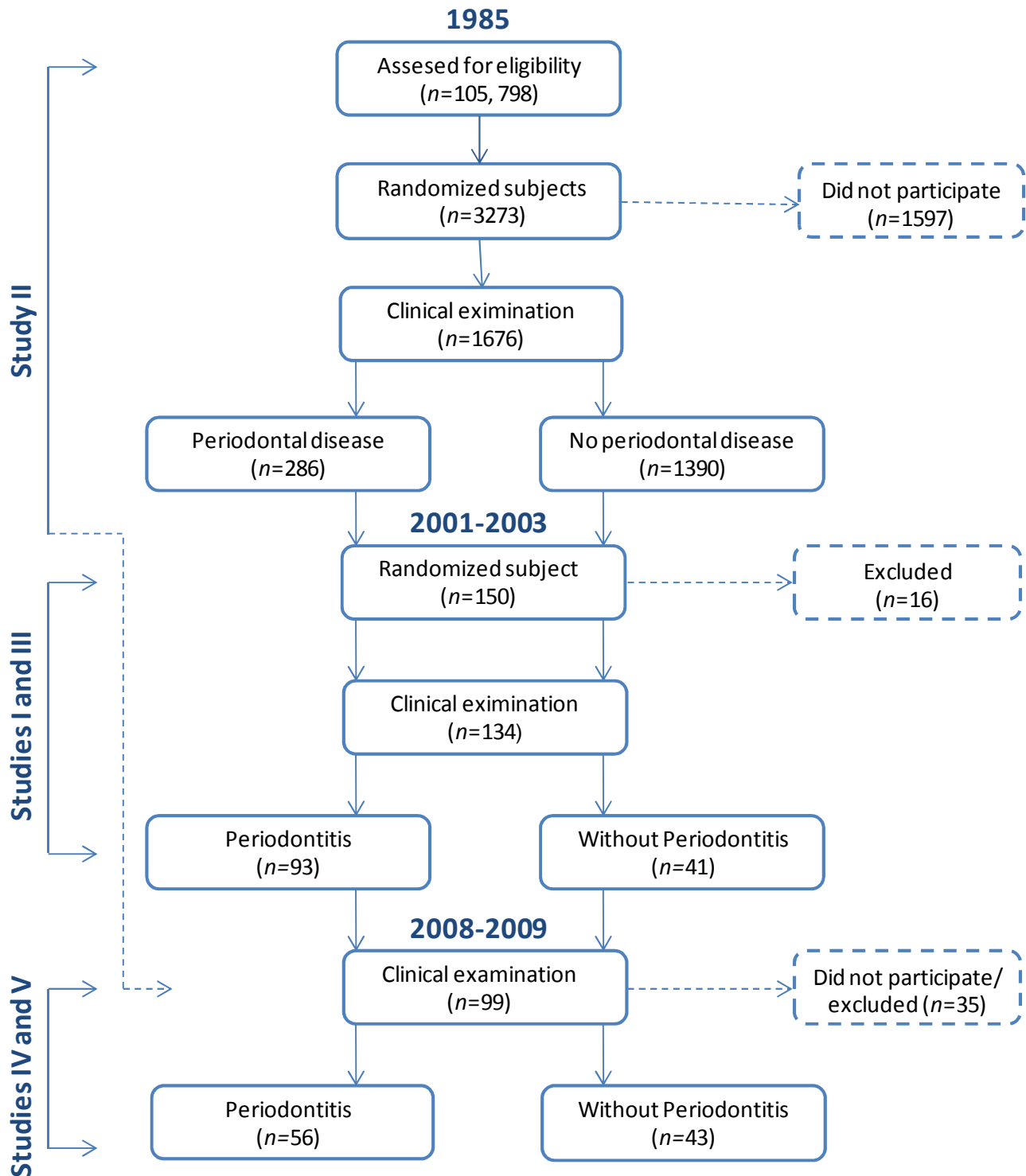


Figure 6. Flow-chart showing the selection of subjects from 1985 to 2009. The clinical examination in 1985 and data from national registry files from 2001 were analysed in 2009 and described in *Study II*. The clinical examination in 2001-2003 was analysed in *Studies I* and *III*, whereas the examination in 2008-2009 is described in *Studies IV* and *V*.

ASSESSMENT OF ORAL HEALTH

All of the clinically examined subjects filled in a medical history report as well as a structured questionnaire concerning health problems, medication, dental visits, tobacco use, marital status, socioeconomic status and education, among others. Overall, the full-mouth clinical examination in 2001-2003 and 2008-2009 included recording the number of remaining teeth (wisdom teeth were excluded due to the frequent occurrence of pseudo-pockets around these teeth), Plaque Index (PLI) (13), Calculus Index (CI) (223), Gingival Index (GI) (14), Bleeding on Probing (BOP), Pocket Depth (PD), and Clinical Attachment Loss (CAL) (30, 31). In 1985, a partial-mouth examination including PLI, CI, GI, and PD, was conducted. A full-mouth set of 14 Kodak Ektaspeed periapical radiographs was obtained from each patient in 2001-2003, and the percentage of remaining bone on radiographs was determined (224) and presented in *Study I*.

Subjects with at least one site with $PD \geq 5$ mm were diagnosed as having periodontal disease in 1985 (*Study II*). In 2001-2003, subjects with three teeth with $PD \geq 5$ mm and BOP were diagnosed as having periodontitis (*Studies I and III*). Subjects with at least one site with $\geq PD$ 5 mm and $CAL \geq 3$ mm were diagnosed as having periodontitis and classified as patients at the examination in 2008-2009 (*Studies IV and V*).

ASSESSMENT OF SUBCLINICAL ATHEROSCLEROSIS

In 2001-2003, antecubital venous blood samples were taken after 12 hours of overnight fasting. HsCRP, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides (TG) were assayed in serum using routine methods at the Laboratory of Clinical Chemistry at Karolinska University Hospital, Huddinge, Sweden (Roche Diagnostics, GmbH, Mannheim, Germany). At the same time, blood pressure was measured and a 12-lead electrocardiogram (ECG) was recorded. Body mass index (BMI) was calculated from anthropometric data as an indicator of overall adiposity. BMI is derived from weight in kilograms divided by square height in meters.

In *Studies I and III*, carotid B-mode ultrasonography was performed between 2001 and 2003. Carotid arteries were examined bilaterally with a duplex scanner (Aspen, Acuson, Mountain View, CA, USA) using a 7 MHz linear array transducer (225). All recordings were carried out by the same trained sonographer. Measurements of distances between the wall echoes within a 10 mm long section of the common carotid

artery were made in late diastole defined by a simultaneous electrocardiographic recording. IMT was defined as the distance between the leading edge of the lumen-intima echo and the leading edge of the media-adventitia echo. The lumen diameter, (LD) was defined as the distance between the leading edge of the intima-lumen echo of the near wall and the leading edge of the lumen-intima echo of the far wall. Carotid plaque was defined as a localized intima-media thickening of greater than 1 mm and at least a 100% increase in thickness compared with adjacent wall segments. To compensate for the stretching effect of arterial distension (secondary to increased arterial pressure) on wall thickness, the cross-sectional intima media area was calculated, cIMA, by using the formula: $3.14 [(lumen\ diameter/2 + intima-media\ thickness)^2 - (lumen\ diameter/2)^2]$ (163).

ASSESSMENT OF BREAST CANCER

For *Study II*, the data for cancer (malignant neoplasm) and causes of death were obtained from the Center of Epidemiology, Swedish National Board of Health and Welfare, Sweden. The data were classified according to the WHO International Statistical Classification of Diseases (ICD) and Related Health Problems. Socio-economic data were obtained from the National Statistics Center, Örebro, Sweden.

The study had a longitudinal prospective design. Many of the subjects had periodontal disease documented at baseline 16 years earlier. The subjects were born on the 20th of any month between 1945 and 1954, which enables diagnosis of BC for each subject up to the year 2001 from the National Cancer Registry.

MICROBIOLOGICAL AND IMMUNOLOGICAL DATA

Sampling and analysis of saliva

In 2008-2009, the subjects were given instructions regarding saliva collections. Saliva samples were collected in restful and quiet circumstances before the clinical examination. To collect unstimulated whole saliva the patient drooled passively into the collection tube for 5 min. For *Study IV*, 0.1 mL of the unstimulated saliva was immediately sent to the laboratory in VMGA transport medium to determine total bacterial count. The samples in VMGA transport medium were diluted and cultured anaerobically on *Brucella* blood agar at 37°C for 7 days (BBL, Becton & Dickinson Company, Maryland, USA). The rest of the saliva was deep-frozen (-70°C) for later analyses.

For *Studies IV* and *V*, salivary immunoglobulin IgA, IgG and IgM concentrations were analyzed by enzyme-linked immunosorbent assay (ELISA) using a plate reader (Labsystems Multiskan RC, Finland) (226). Albumin was analyzed by an immunoturbidometric Tina-Quant® kit (Roche Diagnostics, Switzerland). The kit was modified so that a microtiter plate reader could be used instead of an automatic analyzer (Victor 2 1420 Multilabel Counter, Wallac, Perkin Elmer, Illinois, USA). Salivary total protein was analyzed by a colorimetric method using bovine serum albumin standards and measured spectrometrically at 750 nm (Hitachi U-2001, Japan) (227).

Sampling of GCF

At the examination in 2008-2009, GCF was collected using an intra-crevicular washing technique (**Figure 7**) (77, 228). The test sites were isolated with cotton rolls, gently air-dried, and supragingival plaque was carefully removed. The ejection needle of the instrument was carefully inserted into the crevice to a level of approximately 1 mm below the gingival margin. The sulcus or pocket was then flushed by a constant delivery system (10 µL per flushing) with phosphate-buffered saline and simultaneously drained through the collection needle into Eppendorf tubes by constant suction (flow rate 25 mL/h). The gingival washings in the tubes were diluted up to a final volume of 500 µL by washing the draitubes. The samples were immediately centrifuged (8000 g) for 5 min at 4° C, and the supernatants and pellets were frozen to -70° C until analyses (77, 228).

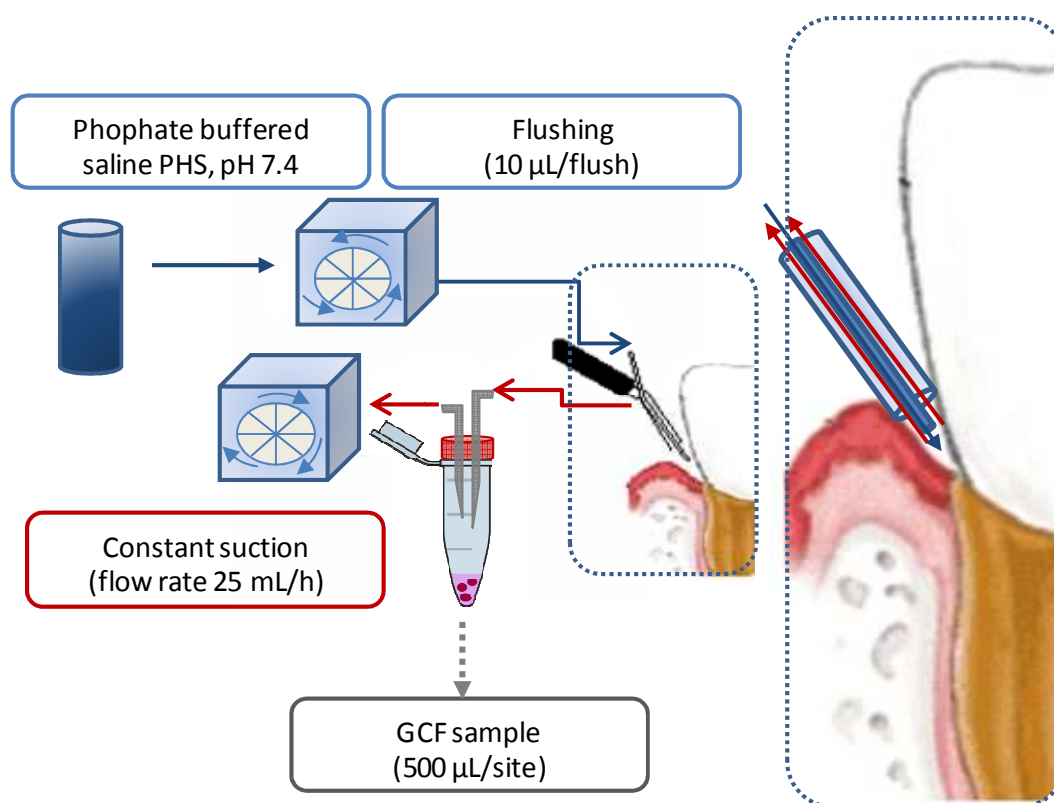


Figure 7. Schematic illustration of the device for gingival crevicular fluid (GCF) collection with an intra-crevicular washing technique. The ejection needle is gently inserted into the sulcus and flushed by a constant delivery system (10 µl per flushing) with phosphate-buffered saline (pH 7.4) and simultaneously drained through the collection needle into Eppendorf tubes by constant suction (flow rate 25 mL/h), and diluted up to a final volume of 500 µl for each test site (77, 228).

Analyses of oral micro-organisms

For *Studies IV* and *V*, the presence of periodontal micro-organisms was measured by polymerase chain reaction (PCR) from the pellet fraction of the GCF samples. Specific primers were designed to hybridize various regions of 16S rRNA genes. *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythia* and *T. denticola*. In brief, DNA was extracted from the GCF-pellets by using a ZR Fungal/Bacterial DNA Kit (Zymo Research, California, USA). An aliquot of 5 µl of DNA suspension was used for the PCR assay of each bacterium. The enzyme used was Dynazyme II Hot Start DNA Polymerase (Finnzymes, Finland). The GeneAmp® PCR System 9700 (Perkin-Elmer Corporation, Massachusetts, USA) was used for the PCR amplification. The PCR products were visualized by UV light after electrophoresis on agarose gel containing ethidium bromide. The limit detection of the PCR method was five to ten cells (229, 230).

Analyses of MMP-8 and MMP-9

For *Studies IV* and *V*, the MMP-8 concentrations in GCF were determined by a time-resolved immunofluorometric assay (IFMA). The monoclonal MMP-8 specific antibodies 8708 and 8706 (Medix Biochemica, Kauniainen, Finland) were used as a catching antibody and a tracer antibody, respectively (98, 231, 232). The GCF MMP-9 levels were determined by ELISA and concentrations were determined according to the manufacturers protocol using commercially available ELISA kits (Amersham Biosciences UK Ltd, Buckinghamshire, UK) (233).

STATISTICAL ANALYSES

The statistical analyses were performed using an SPSS® software package (SPSS Inc. Chicago, IL, USA), version 14.0 (*Study I*) or version 16.1 (*Studies II and III*), and the renamed PASW® Statistics software package, version 18 (PASW Inc. Chicago, IL, USA) (*Studies IV and V*). Differences between data sets at a probability (p) of less than 0.05 were regarded as significant ($p < 0.05$). All p -values are two-tailed, and confidence intervals (CI) were calculated at the 95% level. Results are expressed as mean \pm standard deviation (SD).

In *Study I*, analysis of variance (ANOVA), chi-square tests and multiple logistic regression analysis with backwards elimination of non-significant variables were performed.

In *Study II* ANOVA, chi-square test, Fishers exact t-test, and multiple logistic regression analyses were carried out. The model with the confounders was correlated to the incidences of cancer. A backwards elimination method was used to control for multicollinearity (correlation between confounders).

In *Study III*, significant differences were determined either by Student's unpaired t-test or ANOVA. Chi-square tests were used when analyzing non-parametric data. Multiple logistic regression analysis of hsCRP, with backwards elimination of non-significant variables was performed with a dichotomized median split.

In *Studies IV and V*, Student's unpaired t-test, chi-square test and analyses with Fisher's exact p -value were used. In additional analyses for this thesis, the same tests as already mentioned were performed. Furthermore, a correlation test with Spearman Rho was carried out to analyze which micro-organism that was more prevalent in deep pockets (see **Table 5**).

RESULTS AND DISCUSSION

STUDY POPULATION (STUDIES I-V)

The number of subjects in the five studies is presented in the first table (**Table 1**). The studies consisted of comparable numbers of women and men, except for *Study I*, where we only included data on women (**Table 2**). The women in *Study I* are also described in *Study III*. *Studies IV* and *V* consisted of the same subjects. In all five studies, there was a significant difference in smoking habits between subjects with and without periodontal disease (patient vs. control group), such that more subjects smoked in the patient group than in the control group (*Studies I-V*). The control group had attained a significantly higher education level than the patient group (*Studies I-III*).

Table 1. The number of the subjects in the five studies.

	STUDY				
	I	II	III	IV	V
Participants					
All	67	3273	134	99	99
Women	67	1586	67	46	46
Men	0	1687	67	53	53

Table 2. The number of subjects in the patient and control groups.

	STUDY				
	I	II	III	IV	V
Patient group					
All	46	286	93	56	56
Women	46	125	46	24	24
Men	0	161	47	32	32
Control group					
All	21	1390	41	43	43
Women	21	713	21	22	22
Men	0	677	20	21	21

The patients did not differ regarding the occurrence of hypertension or BMI (*Studies I and III*). In the questionnaire the subjects were asked about any heredity for atherosclerotic disease, but in this regard no difference was observed between the patients and controls (*Study I*). The summary of the reported medical history in 2008-2009 for subjects in *Studies IV and V* revealed that the majority of subjects had no systemic diseases ($n= 71$), two subjects had diabetes type 2, and 9 subjects had high blood-pressure. The most common medication was for high blood pressure ($n= 8$) and hypercholesterolemia ($n= 7$).

The role of lifestyle and risk factors has to be carefully considered and statistically adjusted when studying the link between periodontitis and systemic diseases (119, 152). Several factors have been noted to increase the risk of periodontitis, smoking being at the forefront (18, 28). The subjects in the patient group indeed contained more smokers than the control group; hence, our findings are in agreement with earlier results (28). The statistical analyses were adjusted for shared risk factors such as diabetes mellitus, smoking, education and other relevant risk factors. Further, the study populations in *Studies I-V* were randomly selected from a homogeneous population living in the Stockholm area, Sweden. The subjects were born between the years 1945 and 1954, and thus the limited age range of 10 years allows comparison between the subjects without the bias of age.

ORAL HEALTH FINDINGS (STUDIES I-V)

The results of the oral examination showed that the clinical parameters were significantly different between the patient and control groups. The patient group had significantly more teeth missing than the control group in *Studies I* and *II*. Subjects with periodontal disease also had significantly more molars missing in the mandible than did controls (*Study II*). The patient group had significantly more dental plaque (higher mean PLI), gingival inflammation (higher mean GI) (*Study IV*), deeper pockets (higher mean PD), a higher percentage of bleeding (higher mean BOP), and more loss of attachment (higher CAL) (*Studies I, III-V*). Remaining bone on the radiographs was higher in the control group than in the patient group (*Study I*).

Additional results regarding the relative deep pockets in the subjects examined in 2008-2009 for *Studies IV* and *V*, showed that the patient group had 11.6 mean number of sites with ≥ 5 mm pockets (**Table 3**), and the mean PD of the test sites (second premolars) was between 3.1 to 3.3 mm (**Table 4**).

Table 3. The mean numbers of deep pockets in 2008-2009.

	<i>n</i>	Mean	\pm SD	95% CI
Number of pockets =4mm	97	16.5	10.7	14.7-19.0
Number of pockets \geq 5mm	56	11.6	13.8	7.7-15.5

Table 4. The pocket depth (PD) and number of deep pockets in 2008-2009.

	Mean PD	\pm SD	=4mm	\geq 5mm
Test site 1	3.1	0.9	16	6
Test site 2	3.4	0.9	30	6
Test site 3	3.3	0.8	30	5
Test site 4	3.3	0.8	28	8

Clinical parameters such as PD, CAL, and BOP, are needed to diagnose existing disease, to determine the prognosis and to monitor periodontal disease progression (18, 20, 30). Our findings that the patient group had more PLI, GI, CI, PD, BOP, and CAL are in agreement with previous studies (30, 221).

ATHEROSCLEROTIC FINDINGS (STUDIES I AND III)

The plasma lipid concentrations (mmol/L) and hsCRP (mg/L) levels from the examination in 2001-2003 in the patient and control group are summarized in figure 8. The total cholesterol levels differed significantly between patient and control groups ($5.9 \text{ mmol/L} \pm 0.9 \text{ SD}$, resp. $5.5 \text{ mmol/L} \pm 0.8 \text{ SD}$, $p < 0.05$), otherwise there were no significant differences between the groups regarding levels of LDL, HDL, or TG (**Figure 8**). The levels of hsCRP in the patient group was $2.7 \text{ mg/L} (\pm 3.6 \text{ SD})$, compared with $2.2 \text{ mg/L} (\pm 5.2 \text{ SD})$ in the control group (NS).

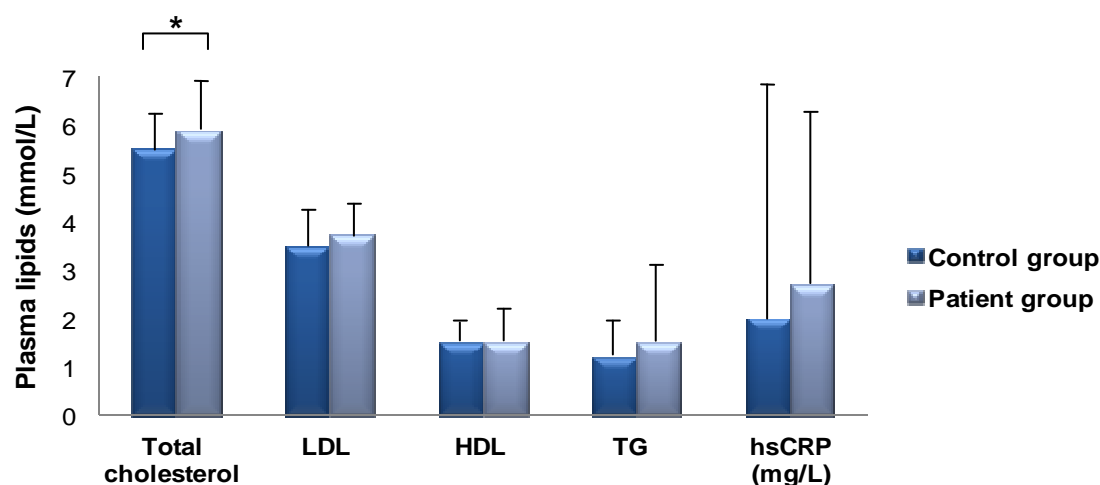


Figure 8. Graph showing the plasma lipid concentrations (mmol/L) and hsCRP levels expressed in mean (\pm SD). Total cholesterol levels was significantly higher in the patient group compared with the control group $^*(p < 0.05)$.

In *Study III*, women had significantly lower hsCRP values and significantly higher HDL cholesterol values than men. Increased levels of hsCRP could not discriminate the patient group from the control group for either men or women. Multiple logistic regression analysis identified age, low HDL cholesterol, and high BMI as the major predictors of high hsCRP value (*Study III*).

The cIMA B-mode variables were significantly greater in the patient group than in the control group, both on the right and left side (**Figure 9**) (*Studies I and III*). Periodontitis appeared to be a principal independent predictor of increased IMT (OR 6.05, 95 % CI 1.34-27.35) and cIMA (OR 5.41, 95% CI 1.20-24.43) in women with periodontitis (*Study I*). Periodontitis was identified as a major independent predictor of cIMA in both women and men (OR 3.82, 95% CI 1.19–12.26) (*Study III*).

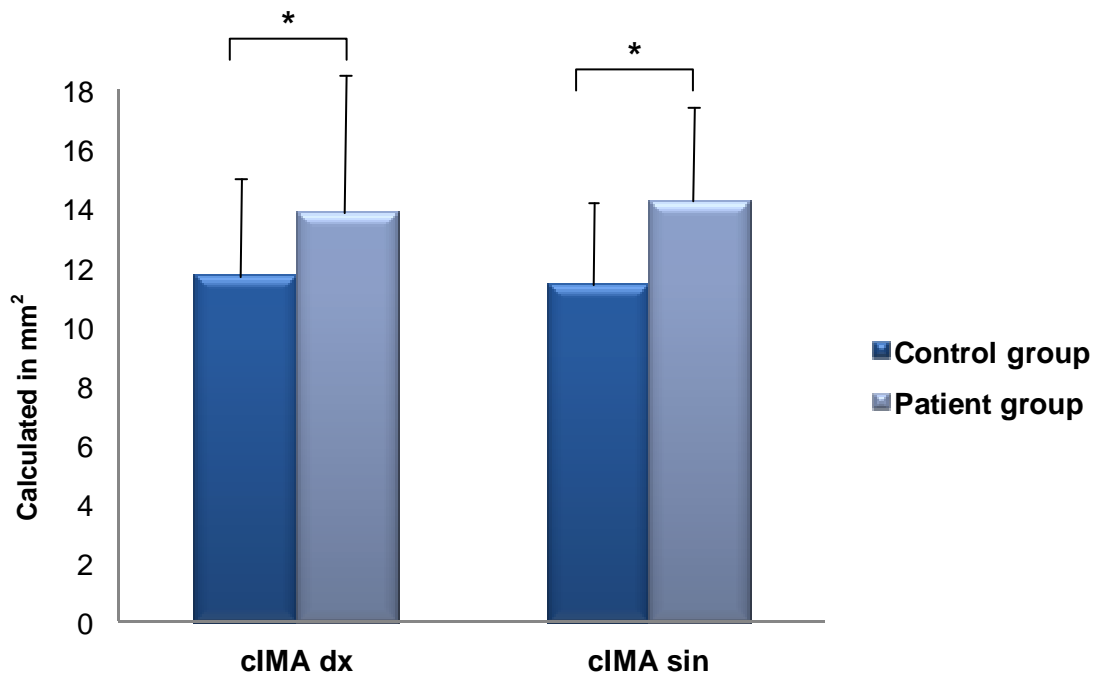


Figure 9. Graph showing the results from carotid ultrasonography and the atherosclerotic lesions expressed in calculated intima-media area (cIMA). The patient group had significantly greater cIMA (mm²) than the control group, at both right (cIMA dx) and left (cIMA sin) site *($p < 0.001$).

The hypothesis was that the chronic inflammatory and microbial burden caused by dental plaque, severe gingival inflammation, and periodontitis would predispose women to the atherosclerotic process (*Study I*). We further hypothesized that chronic infection and inflammation caused by periodontitis would lead to an excessive host response, resulting in a systemic inflammatory reaction. HsCRP levels would thus be increased in subjects with periodontitis, reflecting early vascular atherosclerotic changes (*Study III*). None of the subjects reported any symptoms of overt atherosclerotic disease in 2001-2003 (*Studies I and III*). The results from *Studies I and III* identified periodontitis as a principal independent predictor of carotid cIMA. Indeed, results from numerous studies have indicated that oral diseases, especially, might act as a risk factor for the development of CVD (124, 130, 220). The importance of oral health has been emphasized as a fundamental part of general health. The WHO has also highlighted the importance of integrating oral health in the preventive and health promotion policies (234).

A positive correlation between periodontitis and serum levels of CRP has previously been reported (128). In *Study III*, no association between hsCRP levels and cIMA or periodontitis was found, contrary to our hypothesis. The results from *Study III* showed that women with periodontitis had lower hsCRP concentrations and higher HDL cholesterol than men with periodontitis. Despite high HDL levels, which might exert

significant anti-atherosclerotic effects by decreasing hsCRP levels (235), women with periodontitis might nonetheless be at risk for future cardiovascular events as indicated by the significantly increased cIMA values relative to women without periodontitis (*Studies I and III*). Since gender and periodontitis were two of the independent predictors associated with increased cIMA (*Study III*), we agree with Halvorsen *et al.* (2009) that sex differences should be taken into account in future studies (236).

BREAST CANCER FINDINGS (STUDY II)

In the total cohort of 3273 subjects, 41 subjects were diagnosed as having BC (39 women, 2 men, mean age 45.8 ± 6.8 SD years). Participants diagnosed with periodontal disease and BC, had significantly more missing molars when compared with subjects with periodontal disease but without BC (**Figure 10**). Subjects with any missing molar had an increased incidence of BC in a multiple logistic regression model with BC as the dependent variable (OR 2.36, 95% CI 1.07-5.21). The BC incidence for the clinically examined group ($n= 1676$) showed that 1.75% of those with any missing molar had BC. Subjects with no missing molar had a 0% incidence of BC. The difference in the prevalence of BC in subjects with periodontal disease and with or without any missing molar in the mandible was significant ($p < 0.05$) (*Study II*).

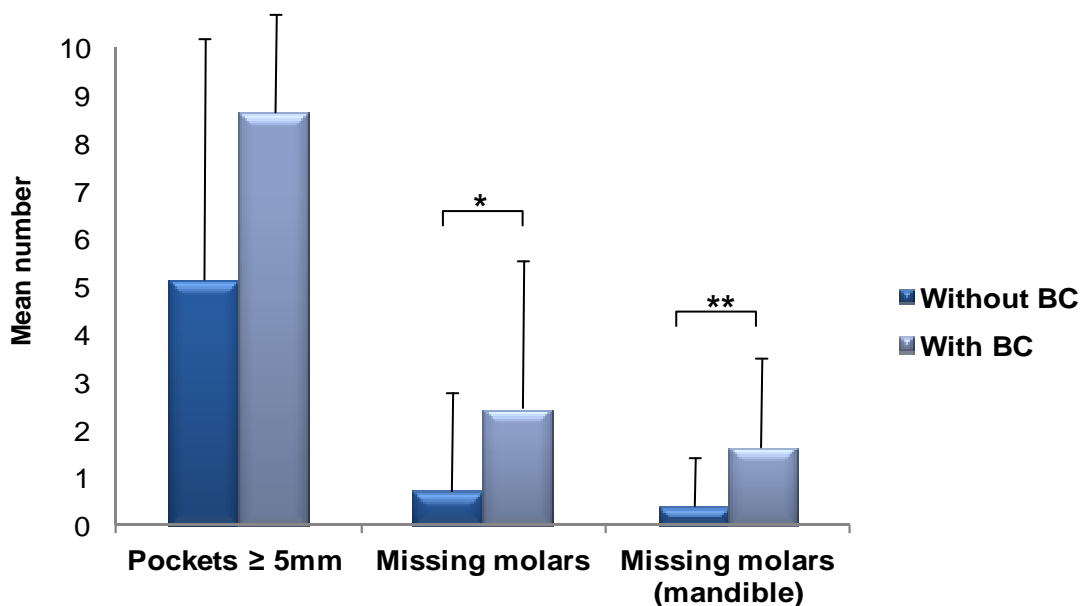


Figure 10. Graph showing some of the clinical oral data for subjects with periodontal disease in 1985. Participants with diagnosed periodontal disease and with BC had significantly more missing molars * ($p = 0.01$), and especially in the mandible ** ($p < 0.005$).

The hypothesis in *Study II* was that a history of low degree of chronic inflammation, such as that seen in periodontal disease, is involved in carcinogenesis, and that the incidence of BC would be associated with periodontal disease and characteristic tooth loss. A few earlier studies have reported an association between periodontitis and cancer (204, 219, 237). The results from *Study II* identified periodontal disease and loss of any molar from the mandible as independent predictors for BC. Michaud *et al.* (2007) reported that a history of periodontal disease was associated with an increased risk of pancreatic cancer, and recent tooth loss was associated with the greatest risk for pancreatic cancer (204). These findings could be considered to be in agreement with the results of *Study II*.

MICROBIOLOGICAL FINDINGS (STUDIES IV AND V)

The patient group was more often infected by the micro-organisms *P. gingivalis*, *P. intermedia* and *T. denticola* than the control group (*Study IV*) when the micro-organisms were detected at \leq one site and expressed as a percentage. However, *T. denticola* was the only micro-organism more frequently found in the patient group than in the control group in site-specific analyses at all four test sites (*Study V*). In additional site-specific analyses, a correlation test (Spearman's Rho) between PD and the presence of the micro-organisms was carried out. The presence of *T. denticola* covaried higher than other micro-organisms with PD, indicating that *T. denticola* was more prevalent in deep pockets, at all four test sites (**Table 5**).

Table 5. Correlation between pocket depth (PD) and presence of *T. denticola*.

	Covaried with PD	
	Spearman's Rho	p-value
Presence of <i>T. denticola</i> at Test site 1	0.22	<0.05
Presence of <i>T. denticola</i> at Test site 2	0.27	<0.01
Presence of <i>T. denticola</i> at Test site 3	0.23	<0.05
Presence of <i>T. denticola</i> at Test site 4	0.27	<0.01

The patient group was more often infected by *P. gingivalis* and *T. denticola* than the control group (*Study IV*). Hence, our findings are partly in agreement with earlier investigations. *P. gingivalis* and *T. denticola* are part of the “red complex” described by Socransky *et al.* (1998), indicating that certain micro-organisms are strongly associated with periodontitis (17). The spirochete *T. denticola* has previously been linked to periodontal disease (238), and was more prevalent at test sites in patients compared with the control group in our study. Furthermore, even if our results are partly in agreement with previous results that have found a higher prevalence of the “red complex” micro-organisms in active sites (17, 42) it must be emphasized that each species has multiple clone types, and only some of these types may be pathogenic (239).

IMMUNOLOGICAL FINDINGS (STUDIES IV AND V)

Salivary flow rate did not differ between the patient and control group. No significant differences existed between the patients and control groups in salivary flow rate or levels of IgA, IgG, IgM, albumin (**Figure 11**), total protein and total bacterial count in saliva (*Study IV*). However, the levels of salivary albumin and total protein were significantly higher in subjects infected with *T. denticola* than in subjects without this bacterium (*Study IV*).

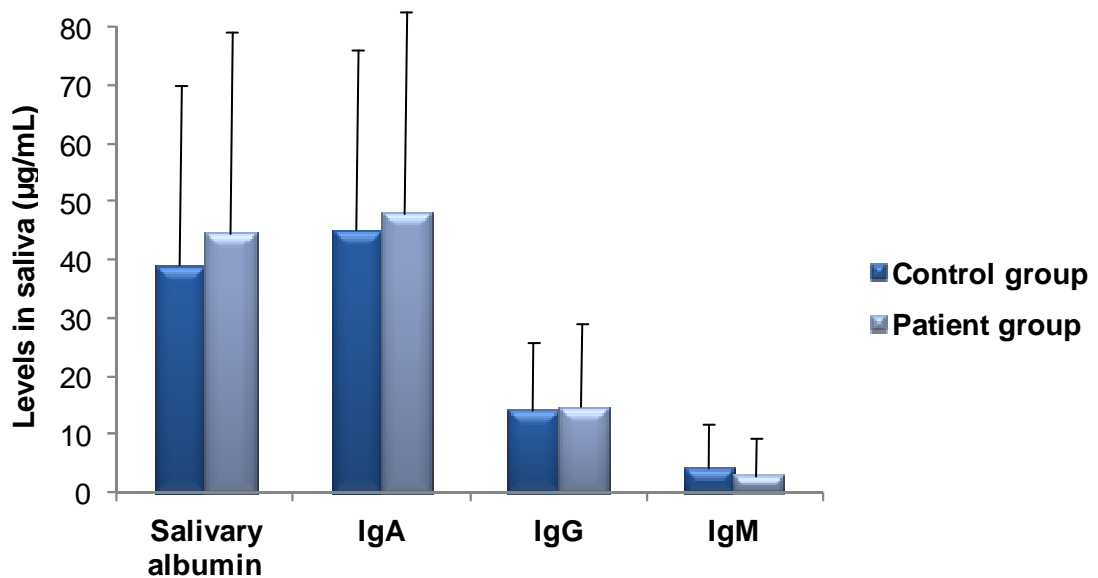


Figure 11. Graph showing the levels of salivary albumin, IgA, IgG and IgM in patient and control group. Levels of salivary albumin, IgA and IgG were slightly higher in the patient group (NS)

The hypothesis was that the presence of specific periodontal pathogens would affect the levels of the biomarkers analyzed (*Study IV*). However, in *Study IV* no significant differences were observed in the levels of IgA, IgG, IgM, salivary albumin and total protein between the patient and control groups. Some studies have reported increased levels of antibodies in subjects with periodontitis, whereas other studies report decreased levels (240). Nevertheless, it must be emphasized that flow rate and composition of saliva differ during the day (241), and the levels of salivary total proteins alter over time (242-244). However, increased levels of albumin and total protein in saliva were associated with the presence of *T. denticola*. Proteins in the periodontal pocket are suggested to be a potential energy sources for the growth of *T. denticola* (245).

MMP-8 AND MMP-9 FINDINGS (STUDIES IV AND V)

A significant increase in MMP-8 levels in GCF with the simultaneous presence of *T. denticola* or *T. forsythia* was observed. These findings were made both in *Study IV* with the presence of micro-organisms at any site and the mean values of MMP-8, and in *Study V* where the site-specific presence of micro-organisms and the levels of MMP-8 were analyzed separately at the four test sites (*Studies IV* and *V*). The site-specific presence of *T. denticola* was also associated with increased levels of MMP-9 in GCF in *Study V*.

The levels of mean MMP-8 and MMP-9 in GCF did not differ significantly between the patient and control groups. However, when analyzing the MMP-8 levels in the four test sites, the mean MMP-8 levels in test site 1 ($n=22$), 2 ($n=35$) and 4 ($n=35$) were higher when the test site had a pocket ≥ 4 mm (**Figure 12**). Moreover, the mean MMP-9 levels in all four test sites were higher when the test site had a pocket ≥ 4 mm than in sites with a PD of < 4 mm (**Figure 13**).

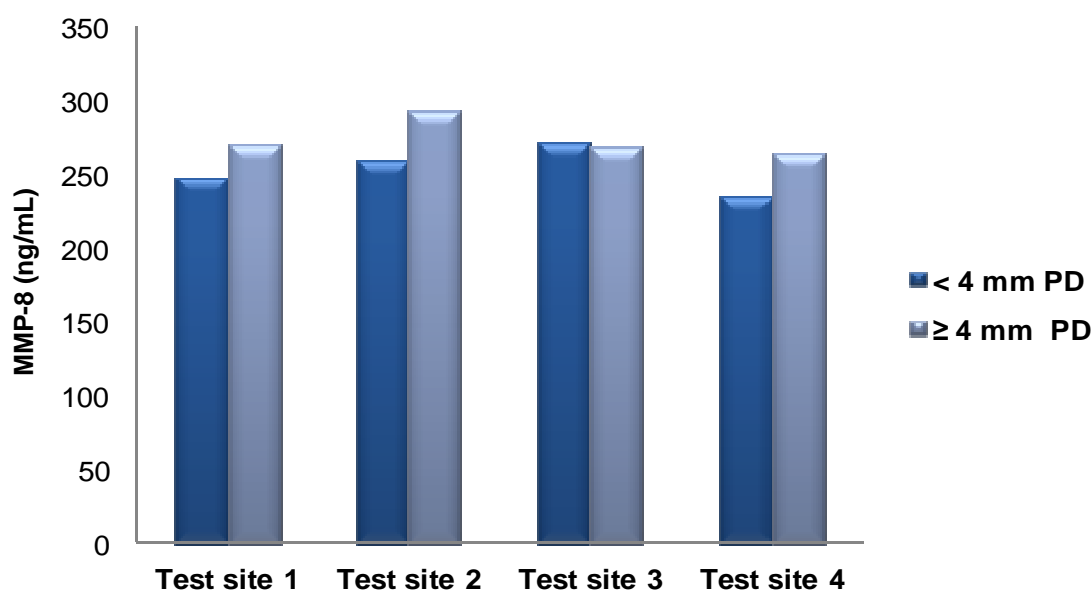


Figure 12. Graph showing the mean levels of MMP-8 (ng/mL) in the four test sites. The mean MMP-8 levels in test 1, 2 and 4 were higher when the test site was ≥ 4 mm PD when compared with sites < 4 mm PD.

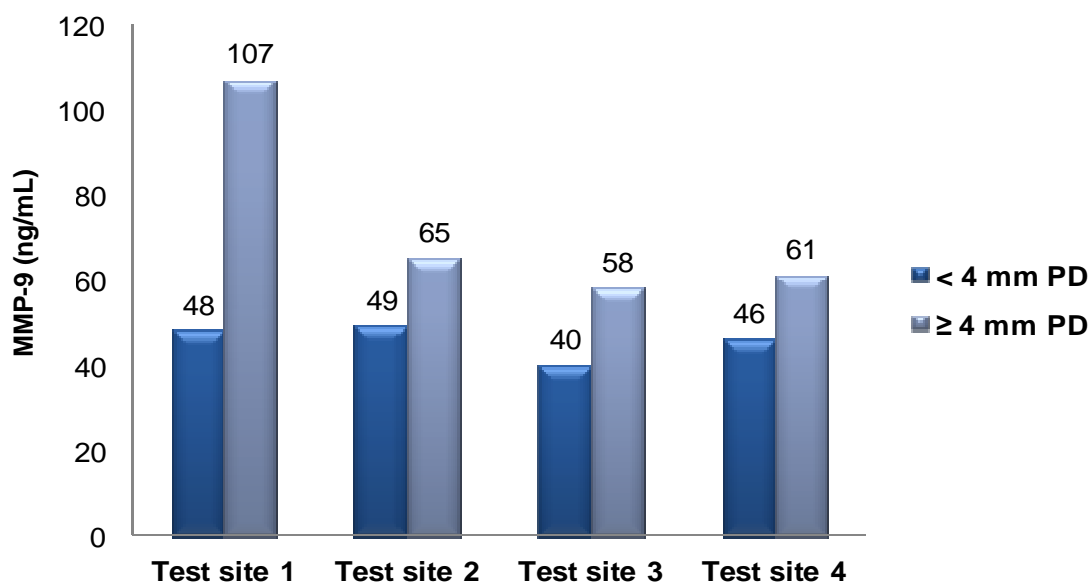


Figure 13. Graph showing the mean levels of MMP-9 (ng/mL) in the four test sites. The mean MMP-9 levels in all test sites were higher when the test site was ≥ 4 mm PD when compared with sites < 4 mm PD.

The hypothesis was that the presence of specific periodontal pathogens would affect the levels of MMP-8 (*Study IV*), and that site-specific presence of these micro-organisms would be associated with increased levels of MMP-8 and MMP-9 (*Study V*). *Studies IV* and *V* confirmed the assumption that periodontal micro-organisms might trigger inflammatory response; the presence of certain micro-organisms associated in elevated levels of GCF MMP-8 and MMP-9. The presence of *T. denticola* was associated with increased levels of MMP-8 and MMP-9, whereas *T. forsythia* was associated with increased MMP-8. The levels of MMP-8 were up-regulated with the simultaneous presence of *T. forsythia* and *T. denticola* in an earlier study by our research group (218). *T. denticola* is known to have numerous virulence factors (246) and to express a serine-type protease that is capable of converting proMMP-8 to the active form of MMP-8 (aMMP-8) (247). Investigations of the interaction between the human host and the bacterial community at the functional level are warranted to elucidate the role of bacteria in the pathogenesis of periodontitis (248).

Our results in *Studies IV* and *V* did not show any significantly different levels of MMP-8 and MMP-9 between the patient and control groups. However, we did find we found that site-specific levels of MMP-8 and MMP-9 were higher in pockets ≥ 4 mm than < 4 mm, and this support the theory of elevated levels of MMP-8 and MMP-9 in periodontal destruction sites. Furthermore, investigating MMP-8 and MMP-9 activities

in GCF site-specifically, as we did in *Study V*, could be a valuable diagnostic aid, complementing the traditional methods (87). Even though levels of MMP-8 in oral fluids are among potential point-of-care periodontitis biomarkers (249) and an increased level of MMP-8 indicates a risk for periodontitis progression, more work is warranted on the prognostic impact of MMP-8 (250). Until now, there has not been a single marker, or combination of markers, that could reliably determine periodontal tissue destruction. Thus, the most reliable method to predict or determine periodontal tissue destruction is clinical measurements (251).

METHODOLOGICAL CONSIDERATIONS

The methodological considerations and confounding factors are discussed more in detail in each of the studies, that can be found in the appendix. This part only provides a brief overview of the important methodological considerations that has to be considered in relation to our findings.

Many definitions of periodontitis have been used in the literature over the years (26, 252, 253), which is also reflected in our studies. Although the classification by Armitage *et al.* (1999) may be useful for clinicians (254), the use of a threshold of sites with deep PD and/or higher CAL is more recommended for standard for population-based studies (253). Regarding the reliability of the obtained results, it can be pointed out that the patients and the controls were randomly chosen in 1985 to avoid selection bias and to ensure normal distribution of the sampled variables.

The carotid sonography was performed and evaluated by the same experienced and blinded sonographer and thus, the methodological biases were minimized. Only individuals with no reported or detected MI were included in 2001-2003; otherwise the atherosclerosis detected by the ultrasound cannot be described as subclinical. Our results on hsCRP are based on accurate and high-sensitivity method to measure CRP that allows the measurement of low levels of CRP (255). Furthermore, the information regarding BC is reliable since it was collected based on national register files.

In a study by Papapanou *et al.* (2000) a high prevalence of the “red complex” micro-organisms were detected in periodontitis patient, but similar high prevalence was furthermore reported in the controls. On the other hand, a substantial difference between patient and controls in colonization of the “red complex” micro-organisms at high levels was revealed by the quantitative analysis of the bacterial load. The authors therefore criticize the use of non-quantitative PCR and a dichotomized categorization, without simultaneous quantification of bacterial load (256). However, the qualitative PCR method we used to detect presence of micro-organisms is rapid, simple and able to detect very small numbers of cells of a given species (229, 230, 257), and therefore the method was adequate, as the aim was to observe the presence of the species. Regarding the data from saliva, the flow rate and composition of saliva varies (241-244), thus repeated measurements on standardized times would have a preference.

GENERAL DISCUSSION AND CONCLUSION

The general aim was to study the association between oral biofilm and certain oral micro-organisms, periodontal disease, and selected inflammatory markers, with a focus on atherosclerosis and BC, in a cohort followed up to 24 years. We selected these systemic disease output variables because of their high prevalence in populations; atherosclerosis remains one of the leading causes of morbidity and mortality while BC leads the incidence of female malignancies (115, 207, 208, 258).

The general hypothesis of this series of studies was that oral infections, in particular periodontal disease, by triggering inflammatory reactions detrimentally affect systemic health where inflammations are known to play a role in the pathogenesis, namely CVD and cancer. Periodontal disease was found to be associated with subclinical atherosclerotic lesions and also a higher incidence of BC. Furthermore, *T. denticola* associated with increased salivary albumin, total protein as well as with higher levels of MMP-8 and MMP-9 in GCF, indicating a possible inflammation triggering capacity of the oral biofilm. Thus, our findings from did confirm our primary hypotheses. The associations indicate that periodontal disease might pose a threat to systemic health.

Our findings regarding the association between periodontitis and early atherosclerotic changes support previous results (259, 260), and may enhance current knowledge by providing a basis for further research, although the studies did not prove causality. Furthermore, we included sufficient controls for the important lifestyle factors that could explain the association between periodontitis and CVD (149). Recently, the American Journal of Cardiology and the Journal of Periodontology published a consensus article about the association between periodontitis and atherosclerotic CVD (120). The consensus concluded that although the inflammation hypothesis presents a conceivable explanation, further research is required to define the mechanism linking periodontitis and CVD (120). Even a modest link between periodontitis and CVD could be of public health importance considering the high prevalence of both diseases, and additional investigations are indeed necessary (122). Established risk factors for CVD have been studied for decades, but defining the relationship between periodontitis and CVD is still in its early stages (119).

Contrary to our hypothesis, hsCRP levels were not associated with either atherosclerotic lesions or periodontitis. Many studies have previously shown that levels of serum CRP are elevated in subjects with periodontitis (186, 187, 260-262), and that CRP levels decrease after periodontal non-surgical treatment (190, 191). Furthermore, CRP levels have been suggested to only increase in some individuals, presumably the ones having a systemic inflammatory reaction (263). The role of CRP as a biomarker in predicting CVD risk is not yet established (173, 264). It remains unclear whether CRP could be used as a marker of exposure or outcome. Other carotid plaque-specific biomarkers, particularly biomarkers with a stronger predictive power for CVD independent of conventional risk factors, are needed for a final conclusion (173). Our findings also emphasize the need for further investigations into the role of hsCRP as a biomarker for subclinical atherosclerosis and periodontitis.

To our knowledge, *Study II* is the first to evaluate the association between periodontal disease and BC. The most comparable findings are in the study by Michaud *et al.* (2007), where a history of periodontal disease was reported to be associated with an increased risk of pancreatic cancer (204). Their study was a prospective questionnaire-based study, whereas our findings are based on a longitudinal prospective design with data from the National Cancer Registry of Sweden. While our results provide a useful basis for hypothesis generation, they need to be confirmed in other large studies. The association between periodontal disease and the incidence of BC may occur through plausible biological mechanisms, but confirmation of this association is necessary before further conclusions can be made (237). More investigations into this association and the role of inflammation are warranted to shed light on our limited understanding of cancer etiology in general (174, 176, 177, 265).

The area of the gingival sulcus or periodontal pocket in patients with periodontitis has been calculated to approximate 8 to 20 cm² (152). This area is especially prone to bacterial dissemination into the systemic circulation. Furthermore, inflammation has the potential to increase cellular proliferation and mutagenesis, inhibit apoptosis, and also increase secretion of inflammatory mediators (175, 201). One potential explanation for the association between periodontal disease and cancer could be that micro-organisms in the oral cavity of subjects with periodontal disease generate systemic inflammation and increased levels of carcinogenic compounds (177). As discussed earlier, specific micro-organisms in the oral cavity have the potential to up-regulate

cytokines and create a prolonged inflammatory environment, such as is the case with periodontal disease (18, 33). Based on our findings, we hypothesize that certain periodontal micro-organisms might trigger an inflammatory response by activating tissue-degrading enzymes such as MMP-8 and MMP-9. Enhanced periodontal tissue destruction involving locally up-regulated and activated gingival tissue and GCF MMPs have indeed been associated to systemic diseases such as CVD (266). Thus, the oral biofilm and micro-organisms may in the future help to demonstrate causality in the link between periodontal disease and systemic diseases such as CVD and cancer.

FUTURE RESEARCH

Treatment strategies for periodontal disease that are less invasive and more cost-effective are needed. Improved molecular techniques for studying the oral biofilm and dental plaque as well as inflammatory cytokines can have therapeutic implications for preventing and treating periodontal disease.

To clarify the link between periodontal disease and systemic diseases, longitudinal studies are required that have a standardized measurement of periodontal disease, a sufficiently long follow up, and a proper adjustment of known confounders. Large controlled trials, where the subjects are randomized to treatment versus standard care of periodontitis, and then followed for CVD events, could advance our understanding of the causality of the link. Such a study design may, however, prove to be ethically problematic. In addition, for the yet unknown links between other diseases that may be associated with periodontal disease, such as cancer, epidemiological studies are warranted to first establish a stronger association.

MAIN FINDINGS

Periodontal disease was found to be associated with subclinical atherosclerotic lesions and also a higher incidence of BC. Furthermore, *T. denticola* associated with increased salivary albumin, total protein as well as with higher levels of MMP-8 and MMP-9 in GCF, indicating a possible inflammation triggering capacity of the oral biofilm. Thus, our findings from the series of studies of this thesis did overall confirm our primary hypotheses. The associations indicate that periodontal disease might pose a threat to systemic health (**Figure 14**). Specifically;

- Our findings indicated that a high amount of dental plaque, severe gingival inflammation and periodontal disease seemed to be associated with the development of atherosclerotic lesions in women already at the early, subclinical stage (*Study I*).
- Chronic periodontal disease, as indicated by missing molars, seemed to be associated statistically with BC (*Study II*).
- HsCRP did not appear to be a sufficiently sensitive marker of the atherogenic process or periodontitis. However, irrespective of low hsCRP levels, periodontal disease might present a risk for atherosclerotic disease, particularly in women (*Study III*).
- The presence of both *T. denticola* and *T. forsythia* associated with increased MMP-8 concentration in GCF. Furthermore, the presence of *T. denticola* associated with increased salivary albumin and total protein concentrations. (*Study IV*).
- The site-specific presence *T. denticola* in GCF appeared to increase the release of MMP-8 and MMP-9 at test sites (*Study V*).

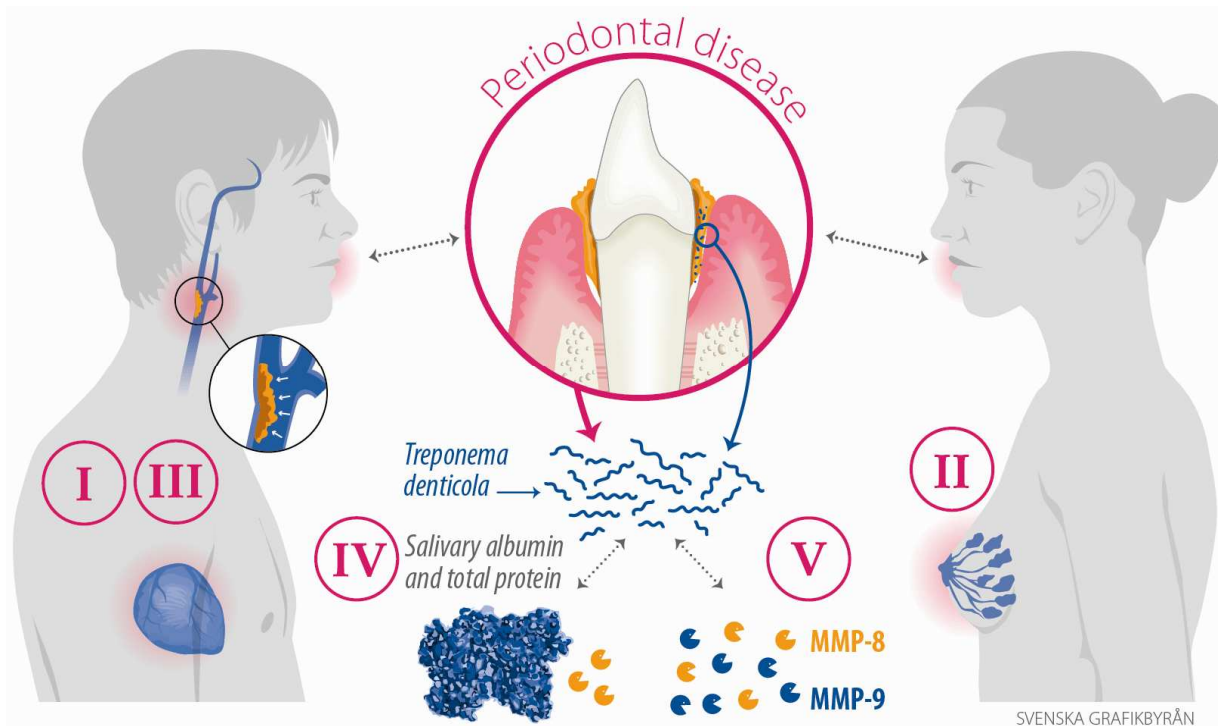


Figure 14. Periodontal disease was found to be associated with subclinical atherosclerotic lesions (*Studies I and III*) and also a higher incidence of breast cancer (*Study II*). *Treponema denticola* associated with increased salivary albumin, total protein as well as higher levels of matrix metalloproteinase, MMP-8 (*Study IV*). Furthermore, the site -specific presence *T. denticola* in GCF appeared to increase the release of MMP-8 and MMP-9 at test sites (*Study V*), indicating a possible inflammation triggering capacity of the oral biofilm. The associations indicate that periodontal disease might pose a threat to systemic health.

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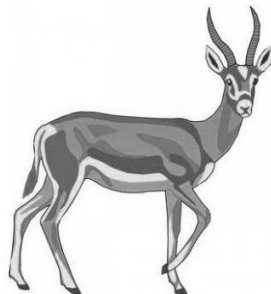
My most sincere appreciation and love to my big family for always supporting me and spoiling me with endless love. I know that as a researcher you have to be very careful when drawing conclusions, but after a comprehensive longitudinal study that started 30 years ago, I can be sure of the following conclusion: I have been blessed with the BEST family ever! My siblings: **George, Philip, Nawal, Tony, Rose, Johnny, Amelin, Laila** and sweet **Nancy**, I adore you all. If I were to write all the things you done for me and how much you mean to me, the acknowledgments part would exceed the length of the entire thesis.... I love you from the bottom of my heart, thank you for being my rock throughout the years! I am also very grateful to my dear “bonus” siblings **Manira, Yolla, Rima, Efgenia, Pierre, Fadi, Elias, Yakub**, that my brothers and sisters chose to marry. Of course, I have to brag about my 21 (until now, “mashalla”...) wonderful nieces and nephews: **Jacob Y, Viktoria, Jack, Michael, Alexander, Ninos, Gabriel, Toni, Rosi, Dani, Marie-Therese, Alex, Jakob H, Antonio, Rikard, Mari-Angelina, Mikael, Kristoffer, David, Jacob K** and finally my one and only goddaughter, charming **Valencia Leonie**! Thank you all for bringing happiness into my life... I love each one of you so much!

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الاهداء الخاص الى من كد وجد من اجلي والدي ووالدتي

إن كتابة هذه الاسطر اليكما يا والدتي العزيزة ويا والدي العزيز كانت من دون ادنى شك الشطر الأكثر صعوبة في هذا العمل. تأبى الانامل ويعجز الاصغر عن شكركما يا من منحني الحياة والحب والسعادة اللامتناهية ناكراً الذات وقاضياً شطراً طويلاً من الحياة جاهداً لتنشئة العائلة الكبيرة وكادحاً لتوفير العلم والتعليم لها. انتما واحد في العطاء، في التضحية، في الكد والجد معا وفي مجابهة الصعاب وتذليلها من اجل تسهيل وعورة الدروب واضاءة نور الامل في الظلمات الداكنة الحالكة. والدتي، إن كلمة الشكر ليس لها اي معنى امام نضالك وكفاحك من اجلنا. اماه يا رمز القوة والجلد مثلك في الصبر والحنان لم ولن اشهد. اماه يا جلدة فوادي انت معي دائما وابدا في كل دقة وفي كل ضربة من ضربات قلبي. والدي العزيز، لقد تركت ذروة النصر وقمة المجد يا ابا المجد، في سبيل إيصال طاقم القارب الى شاطئ الامان، تاركاً الاحبة والاصدقاء، الاهل والاقارب، الدار والديار، العز والجاه، من اجلي ومن اجل بقية افراد العائلة غلبت مصلحتنا على مصلحتك الشخصية مكرساً نفسك لخدمتنا دون كلل او ملل، زائراً وطناً يستوطن فيه مستقبلنا نحن البنون، ولكن سعيك وهدفك ومستقبلك ورفعك قدمتهم شموعاً منيرة تحترق مبينة البياض من السواد في عوالم المهجر البعيدة عن شرفات الاباء والاجداد. ابتاه، شكراً على كل شيء، كلمات الشكر والاعتراف بالجميل مشكلة من احرف مرئية ومقروءة اما ما فعلته من اجلي لا يمكن وصفه بكلمات الالسن واللغات قاطبة. انت رمز الابوة الصالحة والحنان، انت الحكمة بعينها، انت اعظم رجل وطأت قدماه هذه المعمورة العامرة بوجودك وطيبتك اللامتناهية المعالم. لك مني كل الاحترام يا اعظم وانبل من في الدنيا. والدتي العزيزة والدي العزيز انتما بالنسبة لي كل شيء في حياتي ولولاكما لما بلغت هذا القدر من النجاح. استمد منكما القوة والعزيمة دائما وابدا مستلهمة خطوات النجاح من عطاءاتكم اللامحدودة. اشكركما جزيل الشكر لانكما كنتما العضد والسند في رحلة من رحلات العلم لم تكن لتتحقق لولا مؤازرتكما وتشجيعكما لي.

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