TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 1047

MEDICA - ODONTOLOGICA

PREGNANCY AND PERIODONTIUM

A clinical, microbiological, and enzymological approach via a longitudinal study

by

Mervi Gürsoy

TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2012

From the Department of Periodontology and the Postgraduate School of Oral Health Science (PeGaSOS), Institute of Dentistry, University of Turku, Turku; Anaerobe Reference Laboratory, National Public Health Institute (KTL) /current National Institute for Health and Welfare (THL); Biomedicum Research Laboratory, Institute of Dentistry, University of Helsinki; Department of Oral and Maxillofacial Diseases, Helsinki University Central Hospital, Helsinki; and Kerava Municipal Health Care Centre, Kerava, Finland

Supervised by

Professor Eija Könönen
Department of Periodontology,
Institute of Dentistry, University of Turku
Turku, Finland
and
Anaerobe Reference Laboratory,
National Public Health Institute (KTL) /
current National Institute for Health and Welfare (THL)
Helsinki, Finland

Professor Timo Sorsa
Department of Cell Biology of Oral Diseases,
Institute of Dentistry, University of Helsinki
and
Department of Oral and Maxillofacial Diseases,
Helsinki University Central Hospital (HUCH)
Helsinki, Finland

Reviewed by

Professor Tellervo Tervonen
Department of Periodontology and Geriatric Dentistry,
Institute of Dentistry, University of Oulu
Oulu, Finland

Docent Oskari Heikinheimo
Department of Obstetrics and Gynecology,
Helsinki University Central Hospital (HUCH)
Helsinki, Finland

Opponent

Professor Rutger Persson
Guest Professor, Oral Health Sciences
University of Kristianstad
Kristianstad, Sweden
and
Research Professor, Department of Periodontics and Department of Oral Medicine
University of Washington
Seattle, WA, USA
and
Emeritus Professor, Department of Periodontology
University of Bern
Bern, Switzerland

ISBN 978-951-29-5240-3 (PRINT)
ISBN 978-951-29-5241-0 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2012

To my family

ABSTRACT

Mervi Gürsoy. Pregnancy and periodontium – A clinical, microbiological, and enzymological approach via a longitudinal study. Department of Periodontology and the Postgraduate School of Oral Health Science (PeGaSOS), Institute of Dentistry, University of Turku / Anaerobe Reference Laboratory, National Public Health Institute (KTL); currently National Institute for Health and Welfare (THL) / Biomedicum Research Laboratory, Institute of Dentistry, University of Helsinki / Kerava Municipal Health Care Centre. Annales Universitatis Turkuensis. Sarja-Ser D Medica-Odontologica Osa-Tom 1047, Painosalama Oy, Turku, Finland, 2012.

The mechanisms leading to an enhanced susceptibility to gingivitis in pregnant women have not yet been completely described. Therefore, the current study series were performed to investigate longitudinally the influence of pregnancy on periodontal tissues, and to evaluate microbial and host response factors related to pregnancy gingivitis formation.

Pregnancy-related periodontal changes were analysed in 30 generally healthy women (24-35 years old) once per trimester, till the end of lactation. Matched non-pregnant women (n=24) served as the controls, and were examined three times, once per following month. Pregnancy-related gingival inflammation was observed as enhanced tendency towards gingival bleeding and pseudopocket formation with a concomitant decrease in plaque levels. Gingivitis reached its peak during mid-pregnancy and then decreased transiently visit by visit. After lactation, no differences in periodontal status were seen between the study and control populations.

In contrast to previous studies reporting increased levels of *Prevotella intermedia*, a specific aim was to analyse phenotypically two identical species, *P. intermedia* and *Prevotella nigrescens,* separately using a 16S ribosomal DNA-based PCR. As a result, the increased levels of *P. nigrescens* were related to pregnancy gingivitis.

Matrix metalloproteinases (MMPs) are involved in periodontal destruction. However, their role in pregnancy gingivitis is not well studied. Therefore, neutrophilic enzymes and proteinases, such as MMP and myeloperoxidase (MPO) levels were analysed from saliva and gingival crevicular fluid (GCF) samples during the follow-up. Despite increased inflammation and microbial shift towards anaerobes, the host response did not activate the MMP, elastase and MPO secretion during pregnancy.

These results demonstrate that during pregnancy gingival inflammation is enhanced especially during the second trimester, when *P. nigrescens* levels in subgingival plaque were increased, whereas the neutrophilic enzymes and proteinase levels in both saliva and GCF remained low. These findings could explain, at least in part, why pregnancy gingivitis itself does not predispose or proceed to periodontitis.

Key words: female sex hormones, gingivitis, host response, microbes, pregnancy

TIIVISTELMÄ

Mervi Gürsoy. Raskaus ja parodontium – Kliininen, mikrobiologinen ja entsymologinen näkökulma. Parodontologian osasto ja PeGaSOS-tutkijakoulu, Hammaslääketieteen laitos, Turun yliopisto / Anaerobibakteerilaboratorio, Kansanterveyslaitos (KTL); nykyinen Terveyden ja hyvinvoinnin laitos (THL) / Biomedicum tutkimuslaboratorio, Hammaslääketieteen laitos, Helsingin yliopisto / Keravan terveyskeskus. Annales Universitatis Turkuensis. Sarja-Ser D Medica-Odontologica Osa-Tom 1047, Painosalama Oy, Turku, Suomi, 2012.

Raskaudenaikaisen ientulehdusherkkyyden lisääntymiseen johtavia mekanismeja ei ole vielä täydellisesti kuvattu. Tässä pitkittäistutkimussarjassa selvitettiin raskauden vaikutusta hampaiden kiinnityskudoksiin sekä tutkittiin mikrobien ja isännänvasteeseen liittyvien tekijöiden vaikutusta ientulehduksen muodostumisessa.

Raskauden vaikutusta hampaiden kiinnityskudoksiin tutkittiin 30 (24-35-vuotiaalta) yleisterveeltä naiselta kerran jokaisen raskauskolmanneksen aikana sekä synnytyksen ja imetyksen päättymisen jälkeen. Samankaltaiset, ei-raskaana olevat naiset (n=24) toimivat verrokkeina, ja heidät tutkittiin peräkkäisinä kuukausina yhteensä kolme kertaa. Samanaikaisesti plakin määrän laskiessa raskaudenaikainen ientulehdus lisääntyi, mikä havaittiin kohonneena ienverenvuotona ja ientaskumuodostuksena ilman todellista kiinnityksen menetystä. Raskauden puolivälissä ientulehduksen esiintyvyys saavutti huippunsa, mikä tämän jälkeen laski käynti käynniltä saavuttaen matalimman tasonsa imetyksen päättymisen jälkeen, jolloin tulehduksen määrä vastasi ei-raskaana olevien naisten tasoa.

Toisin kuin aiemmissa tutkimuksissa, joissa on raportoitu *Prevotella intermedia* -määrien lisääntyvän raskauden aikana, tämän tutkimuksen erityisenä tavoitteena oli erotella toisistaan ilmiasultaan kaksi samanlaista lajia, *P. intermedia* ja *Prevotella nigrescens*, 16S ribosomi-DNA-PCR –menetelmän avulla. Tutkimuksessa selvisi, että kohonneet *P. nigrescens* -määrät liittyivät raskaudenaikaiseen ientulehdukseen.

Vaikka matriksimetalloproteinaasit (MMP:t) liittyvät hampaiden kiinnityskudostuhoon, niiden roolia raskaudenaikaisessa ientulehduksessa ei ole juurikaan tutkittu. Näin ollen, neutrofiiliperäisten entsyymien ja proteinaasien, kuten esimerkiksi MMP:n ja myeloperoksidaasin (MPO) tasot analysoitiin syljestä ja ientaskunesteestä. Huolimatta ientulehduksen ja anaerobilajien määrän lisääntymisestä, isännänvaste ei aktivoinut MMP-, elastaasi- ja MPO-eritystä raskauden aikana.

Nämä tulokset osoittavat, että ientulehduksen määrä lisääntyy varsinkin raskauden toisella kolmanneksella, jolloin *P. nigrescens* -tasot subgingivaalisessa plakissa kohoavat, kun taas neutrofiiliperäisten entsyymien ja proteinaasien tasot pysyvät alhaisina sekä syljessä että ientaskunesteessä. Nämä havainnot voivat ainakin osittain selittää, miksi raskaudenaikainen ientulehdus ei itsessään altista tai johda parodontiittiin.

Avainsanat: naishormonit, ientulehdus, isännänvaste, mikrobit, raskaus

TABLE OF CONTENTS

ABBREVIATIONS

BOP	bleeding on probing
CAL	clinical attachment level
CEJ	cemento-enamel junction
CFU	colony forming unit
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
EYA	egg yolk agar
GCF	gingival crevicular fluid
GI	gingival index
IFMA	time-resolved immunofluorometric assay
IL	interleukin
KVLB-2	kanamycin vancomycin laked blood agar
LPS	lipopolysaccharide
MMP	matrix metalloproteinase
MPO	myeloperoxidase
N-Pr	non-pregnant control population
OPG	osteoprotegerin
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
PlI	plaque index
PMN	polymorphonuclear leukocyte, neutrophil
PD	pocket depth
PPD	probing pocket depth
Pr	pregnant study population
RANKL	receptor activator for nuclear factor κB ligand
RLB	rabbit laked blood agar
rDNA	ribosomal deoxyribonucleic acid
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TIMP	tissue inhibitor of matrix metalloproteinase
TNF-α	tumor necrosis factor-alpha
VPI	visible plaque index

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by their Roman numerals **(I-IV)**:

I Gürsoy M, Pajukanta R, Sorsa T, Könönen E. Clinical changes in periodontium during pregnancy and post-partum. *J Clin Periodontol* 2008;35:576-83.

II Gürsoy M, Haraldsson G, Hyvönen M, Sorsa T, Pajukanta R, Könönen E. Does the frequency of *Prevotella intermedia* increase during pregnancy? *Oral Microbiol Immunol* 2009;24:299-303.

III Gürsoy M, Könönen E, Tervahartiala T, Gürsoy UK, Pajukanta R, Sorsa T. Longitudinal study of salivary proteinases during pregnancy and post-partum. *J Periodontal Res* 2010;45:496-503.

IV Gürsoy M, Könönen E, Gürsoy UK, Tervahartiala T, Pajukanta R, Sorsa T. Periodontal status and neutrophilic enzyme levels in gingival crevicular fluid during pregnancy and post-partum. *J Periodontol* 2010;81:1790-6.

Additionally, some unpublished data are presented.

1. INTRODUCTION

Changes in female sex hormone levels during pregnancy are related to the enhanced susceptibility to gingival inflammation. This phenomenon, also named pregnancy-related gingivitis, typically occurs without a clear association with the amount of dental plaque, and develops more severe forms, in comparison to plaque–induced gingivitis in non-pregnant women (Silness and Löe, 1964; Cohen *et al*., 1971). Clinically, pregnancy-related gingivitis is usually seen as an increased tendency towards gingival bleeding and deepened pocket depth. Pregnancy-related gingivitis is a relatively common phenomenon; the prevalence rate has been reported to vary from 35 to 100 % in different ethnic populations (Hasson, 1960; Löe and Silness, 1963; Hugoson, 1971).

Female sex hormones have a multifunctional influence on the periodontal condition in different parts of the life cycle in women. These hormones also play an important role in the pathogenesis of pregnancy-related gingivitis. Increased sex steroid hormone levels can cause edema in tissues by increasing the vascular permeability and cellular proliferation in blood vessels (Lindhe and Brånemark, 1967; Lindhe *et al*., 1967). In addition to their direct physiological effects on periodontal tissues, elevated estradiol and progesterone concentrations during pregnancy alter the quality of subgingival microbiota towards a higher anaerobe-aerobe ratio by favouring the growth of certain gram-negative anaerobes in the oral cavity (Kornman and Loesche, 1980; Jensen *et al*., 1981). Furthermore, these hormones are able to reduce the host response in various ways.

As mentioned above, there seem to be a number of depleting mechanisms decreasing the periodontal tissue resistance in the altered hormonal state during pregnancy. So far, however, neither the etiology of pregnancy gingivitis nor periodontal defence mechanisms during pregnancy have been clearly defined. Thus, more data on the combined effects of these factors and mechanisms are needed. This is important, since the mother's oral health may affect the child both during pregnancy and post-partum. Firstly, an improved knowledge of periodontal changes occurring in generally and periodontally healthy, non-smoking pregnant women will help to clarify the underlying mechanisms related to poor periodontal health and possible adverse pregnancy outcomes (Wimmer and Pihlstrom, 2008; Kunnen *et al*., 2010). Secondly, after birth, the mother's saliva is the most likely vehicle for oral bacteria transmission from mother to child (Könönen *et al*., 1992; 1994).

On the basis of this background, the current comprehensive study series, using clinical, microbiological, and host response-related methods, was performed to provide an overview of pregnancy gingivitis, and obtain updated information about periodontal changes during pregnancy.

2. REVIEW OF THE LITERATURE

2.1 HEALTHY PERIODONTIUM

The periodontium, which is formed of gingiva, periodontal ligament, root cementum and alveolar bone, is a functional unit attaching each tooth to the jaw and supplying support around the teeth. In addition, the periodontium contributes protective roles by sharing the mastication forces and working as a defence barrier against masticatory friction and microbes. Under clinically healthy conditions, the periodontium sustains its consistency of structure and function by keeping the balance between degradation and regeneration, without any clinical signs of gingival inflammation (*i.e.* redness, swelling, bleeding or exudate) **(Figure 1)**.

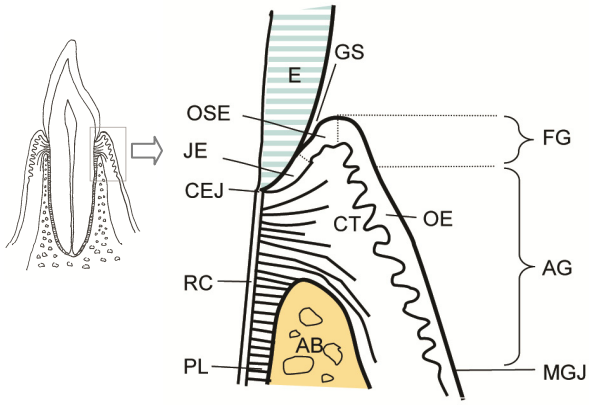


Figure 1. The structure of a healthy periodontium

Gingiva is a part of the masticatory mucosa, which conceals the alveolar bone and encircles the cervical part of the tooth. Gingiva is formed of attached and free gingiva. The attached gingiva continues from the cemento-enamel junction (CEJ) to the mucogingival junction. The free gingiva (marginal and interdental) is coral or pink in colour, has a firm consistency, and extends from the coronal tip of the soft tissue margin to the CEJ (Hassel, 1993; Schroeder and Listgarten, 1997). The shallow space between the free gingiva and the tooth, which has a depth of 1.5-2 mm in healthy conditions, is called the gingival sulcus. In germ-free conditions, no gingival sulcus exists; however, clinically, a gingival sulcus with a depth of less than 3 mm, associates with periodontal health.

Structurally, the free gingiva comprises three different subtypes of epithelium and the underlying connective tissue, lamina propria, which contains various collagen fibre bundle groups, blood and lymphatic components and nerves. The epithelial outer layer facing to the oral cavity includes keratinised oral epithelium, whereas the non-keratinised or seldom parakeratinised oral sulcular epithelium forms the epithelial wall of the gingival sulcus. The junctional epithelium is a thin, non-keratinised epithelium, which contains wide intercellular spaces and a low number of desmosome connections. It forms the epithelial attachment

between the tooth and gingiva, which is strategically an important border between the host and microbes. As a result of the highly porous structure, it provides an easy transit passage for polymorphonuclear leukocytes (PMNs) through the epithelium into the gingival sulcus. The junctional epithelium also plays a remarkable role in the innate defence by secreting chemokines and proinflammatory cytokines (Bosshardt and Lang, 2005).

The periodontal ligament is a densely vascular and cellular connective tissue apparatus, which encircles the root and anchors the tooth to the alveolar socket by joining the root cementum to the bone. The main component of the collagenous fibre bundles is type I collagen with a lesser amount (15-20%) of type III collagen (Hassell, 1993). The periodontal ligament cells are able to differentiate into osteoblasts or cementoblasts (Nojima *et al*., 1990), and they play a significant part in the maintenance of the periodontal tissue integrity (Somerman *et al*., 1990).

The cementum is a mineralised and avascular tissue, which covers the external part of the root and provides a suitable attaching surface to the periodontal ligament. There is no physiologic resorption or remodelling in the cementum; however, the deposition of new cement progresses at variable rates during the lifetime (Hassell, 1993).

The alveolar bone is a tooth-dependent structure; the alveolar socket is formed during tooth formation and eruption, and is sustained until the tooth is lost. The alveolar process undergoes active bone resorption and bone formation in response to several local and systemic factors. In fact, this continuous bone remodelling makes the alveolar bone less stable, which in turn might have some importance in the pathogenesis of periodontitis.

2.2 GINGIVAL AND PERIODONTAL DISEASES

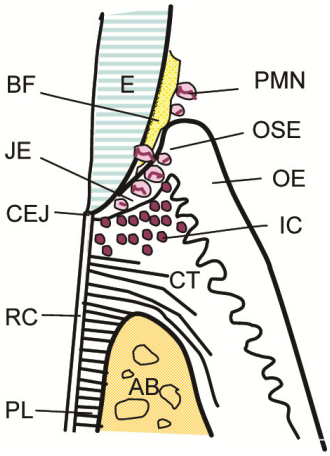
The latest periodontal disease classification has been outlined and updated in the International Workshop for a Classification of Periodontal Disease and Conditions by the American Association of Periodontology in 1999. Instead of age or disease progression rate, the concept of this version is merely based on the infection and host response paradigm by reflecting our present knowledge about periodontal diseases (Armitage, 2002). An overview of the classification system is presented in **Table 1.** At the moment, the classification is based on two main groups, gingival diseases and periodontal diseases (Armitage, 1999). As the current classification is still not solidly based on etiological and pathogenic features of periodontal diseases, it is expected to be revised and modified, whenever suitable new evidence-based data are available (Armitage, 2002).

Table 1. Classification of periodontal diseases and conditions (modified from Armitage, 1999)

I Gingival diseases	II Periodontal diseases
1. Dental plaque-induced gingival diseases	1. Chronic periodontitis
2. Non-plaque-induced gingival lesions	2. Aggressive periodontitis
	3. Periodontitis as a manifestation of systemic disease
	4. Necrotizing periodontal diseases
	5. Abscesses of the periodontium
	6. Periodontitis associated with endodontic lesions
	7. Developmental or acquired deformities and conditions

2.2.1 Plaque-induced gingivitis

The most common form of gingival diseases is plaque-induced gingivitis. According to the results of the Health 2000 Survey, 64% of Finnish women aged 30-34 years had gingivitis (Knuuttila and Suominen-Taipale, 2008). The initiation of gingivitis requires a sufficient amount of bacteria to adhere, colonise and form biofilms on tooth surfaces. Furthermore, as long as the exposure to bacterial biofilm persists, it enables the gingival inflammation to be enhanced. As a response to apically directed microbial growth, proliferation of junctional epithelium into connective tissue, increased infiltrates of leukocytes and plasma cells, and enhanced neutrophil emigration occur (**Figure 2)**. Clinically, gingival redness, bleeding, edema, and increased gingival crevicular fluid (GCF) flow are also seen. In smokers, however, these clinical signs are often reduced or even absent.



AB = alveolar bone
BF = biofilm
CEJ = cemento-enamel junction
CT = connective tissue
E = enamel
IC = inflammatory cells
JE = junctional epithelium
OE = oral epithelium
OSE = oral sulcular epithelium
PL = periodontal ligament
PMN = polymorphonuclear leukocyte, neutrophil
RC = root cementum

Figure 2. Schematic description of plaque-induced gingivitis

In gingivitis, the inflammation is limited to the subepithelial region, thus, neither attachment loss nor alveolar bone resorption occurs. The inflammatory changes in gingiva are reversible, whenever bacterial biofilm is removed.

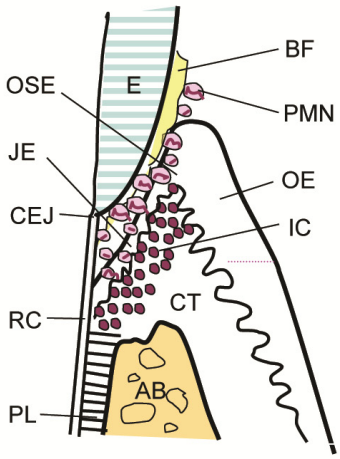
Gingivitis may continue for several years without proceeding to periodontitis (*i.e.* periodontal tissue destruction). However, at susceptible sites, repeatedly measured gingival bleeding (Schätzle *et al*., 2003) and high percentages of bleeding on probing (BOP) >20%) (Lang *et al*., 1986; Joss *et al*., 1994) are considered to be risk factors for further tissue destruction and the development of chronic periodontitis. In fact, gingival bleeding is an initial tissue response against subgingival periodontal bacteria already in shallow gingival pockets, whereas periodontal health-related bacteria reveal an opposite association with gingival bleeding and pocket depth (PD) (Demmer *et al*., 2008).

2.2.2 Chronic periodontitis

The progression from prolonged gingival inflammation to chronic periodontitis is seen as apical migration of the junctional epithelium, releasing the contact point with CEJ, leading

to loss of attachment and periodontal pocket formation (Pirilä *et al*., 2003; Bosshardt and Lang, 2005). Additionally, alveolar bone loss is evident already at early phases of the disease **(Figure 3)**. Usually the disease development is slow, but rapid periods of tissue destruction may also occur (Goodson *et al*., 1982).

A diagnosis of periodontitis is defined as when the probing pocket depth (PPD) is ≥4 mm in at least two non-adjacent teeth, and proximal attachment loss is ≥3 mm (Tonetti and Claffey, 2005; Page and Eke, 2007). The extent of chronic periodontitis is characterised as localised or generalised based on the percentage of affected sites, ≤30% or >30%, respectively (Armitage, 1999). Furthermore, the severity of the disease is classified as slight, moderate, or severe according to the degree of attachment loss. According to the results of the Health 2000 Survey, 40% of Finnish women aged 30-34 years had at least one periodontal pocket of ≥4 mm (Knuuttila and Suominen-Taipale, 2008).



AB = alveolar bone
BF = biofilm
CEJ = cemento-enamel junction
CT = connective tissue
E = enamel
IC = inflammatory cells
JE = junctional epithelium
OE = oral epithelium
OSE = oral sulcular epithelium
PL = periodontal ligament
PMN = polymorphonuclear leukocyte, neutrophil
RC = root cementum

Figure 3. Schematic description of chronic periodontitis

2.3 PATHOGENESIS OF PERIODONTAL DESTRUCTION

The balance of the host-microbial interaction in the healthy periodontium must be interrupted before periodontitis develops and proceeds. First of all, a shift in the subgingival biofilm, seen as an altered balance between health-associated or commensal and pathogenic microbes, is needed (Ximenez-Fyvie *et al*., 2000; Kumar *et al*., 2006; Tanner *et al*., 2006; Teles *et al*., 2010). However, bacterial infection itself is not enough to cause periodontal tissue breakdown. The degradation of tooth-supporting tissues requires impairment of the multifactorial relationship between the host and periodontal pathogens, with the addition of several modifiable and non-modifiable risk factors **(Figure 4)**.

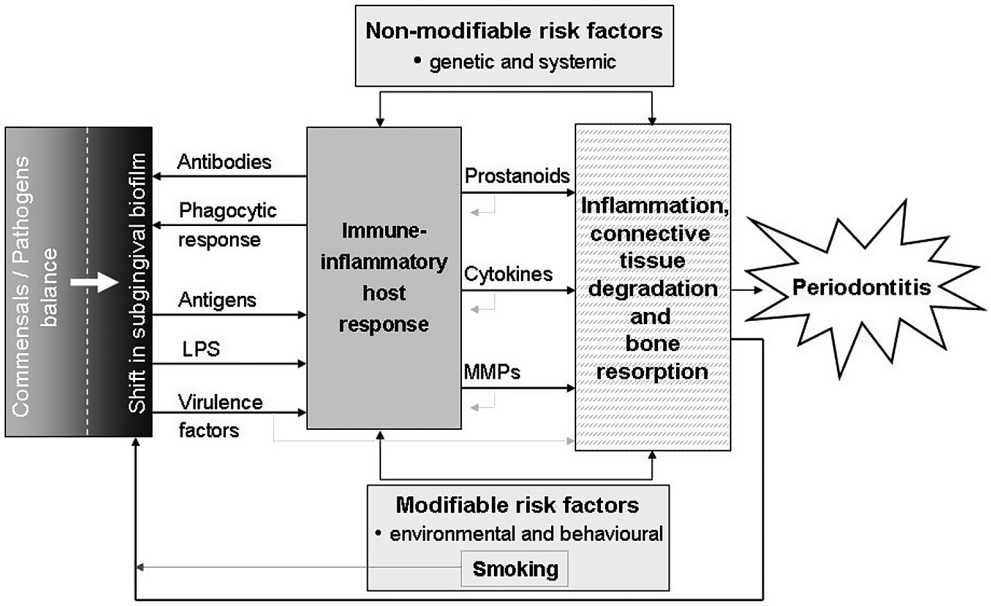


Figure 4. The pathogenesis of periodontitis. (Modified from Page and Kornman, 1997).

2.3.1 Shift in the oral microbiota

There are approximately 600 different bacterial species or phylotypes in the oral cavity (Dewhirst, *et al.* 2010). These oral microbes do not survive for long in planktonic forms, and thus, they preferably adhere to the surfaces and aggregate with each other to form biofilms, where they survive, multiply, and initiate diseases (Jakubovics and Kolenbrander, 2010). Interestingly, even in healthy condition, the oral cavity contains a highly diverse microbiota, which is site- and subject-specific (Aas *et al.*, 2005). Despite this microbial diversity, however, very few changes in the subgingival biofilm have been found among periodontally healthy subjects (Kumar *et al*., 2006). Therefore, a stable microbiota might be a potential predictor of periodontal stability, whereas the shifts in the microbial composition are commonly accompanied by clinical periodontal changes (Ximenez-Fyvie *et al*., 2000; Kumar *et al*., 2006; Tanner *et al*., 2006; Teles *et al*., 2010). Potential effectors to interrupt the balance are thought to be the environmental, behavioural, and endogenous factors, which also affect the host defence. However, the underlying mechanisms of this shift still need to be defined.

The vast complexity of the subgingival microbiota limits the possibilities to precisely describe the microbial etiology of periodontitis. However, the association between the microbial shift and disease initiation is related, at least in part, to the quantity and quality of the microbes in the gingival sulcus. In a clinically healthy gingival crevice, the number of cultivable bacteria is much less (approximately 10^3-10^4 cells) in comparison with the diseased site with a high number of microbes (up to 10^7-10^8 cultivable bacterial cells). In addition, its pathogenic biofilm is formed of a more diverse bacterial community in comparison with a

health-associated biofilm. Thus, enrichment of certain potential pathogens in the subgingival microbiome is needed to initiate the periodontal changes.

Etiologically the most important periodontal microorganisms are gram-negative, facultative or obligatory anaerobic rods. Traditionally, *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* have been considered the key organisms of periodontitis, but also *Campylobacter rectus*, *Fusobacterium nucleatum*, and *Prevotella intermedia* are commonly found in periodontitis (Socransky *et al*., 1998; Könönen *et al.*, 2007; Teles *et al*., 2010). These periodontal pathogens release various bacterial antigens and potential virulence factors, including proteolytic enzymes, leukotoxins, endotoxins (lipopolysaccharide (LPS) of gram-negative bacteria), which in turn, provoke the host cells to produce the first-line inflammatory mediators. Consequently, the over-expressed defence activation of the host leads to periodontal tissue breakdown at the cost of protection.

2.3.2 Immune-inflammatory host response

The elements of the innate and adaptive immune system are described in detail in **Figure 5.** Thanks to its high renewal capability, cell-to-cell junctions, and anti-microbial peptide production, the epithelium forms a strong and active barrier against the constant bacterial challenge of the oral cavity (Schroeder and Listgarten, 1997). However, if the primary defence mechanisms at organ level are failed, the pathogens manage to penetrate the periodontal tissues. This leads to the activation of the specific cellular and molecular mechanisms of the innate immune system (Garlet *et al.*, 2006; Trombone *et al*., 2009). Therefore, enhanced microbial load and bacterial by-products in the gingival sulcus trigger the epithelial cells, and also fibroblasts in the underlying connective tissue, to initiate the proinflammatory cytokine secretion, such as interleukin (IL)-1, -6, -8 and tumor necrosis factor-alpha (TNF-α), growth factors (e.g. epidermal growth factor), proteases (matrix metalloproteinase (MMP) -2, -7, -9, -13), and natural antimicrobial peptides (β-defensin, cathelicidin LL-37) (Bosshardt and Lang, 2005). These inflammatory mediators stimulate PMNs (also called neutrophils) to migrate and increase their presence in the gingival sulcus area, where they form a barrier between the host tissue and microbes (Tonetti *et al.*, 1998). Once the bacteria bind to the neutrophil surface, PMNs begin the phagocytosis, *i.e*. the elimination of the bacteria by entrapment of bacteria into a membrane-delimited structure, the phagosome (Faurschou and Borregaard, 2003; Klebanoff, 2005). Bacteria within the phagosome and phagolysosome may be killed by oxidative or non-oxidative mechanisms. Non-oxidative mechanisms of killing involve phagosome-lysosome fusion, resulting in the secretion of anti-bacterial substances such as lysozyme, MMPs, cathepsin G, PMN elastase, other serine proteinases, and α-defensins (Odeberg and Olsson, 1976; Faurschou and Borregaard, 2003; Klebanoff, 2005). Immediately after PMNs come into contact with microbes, myeloperoxidase (MPO) as an extremely potent microbicidal agent is released into the phagosome or extracellular space (Odeberg and Olsson, 1976; Faurschou and Borregaard, 2003; Klebanoff, 2005). Furthermore, a serine protease, PMN elastase, is able to improve this antimicrobial activity by degradation of the microbial compounds (Odeberg and Olsson, 1976).

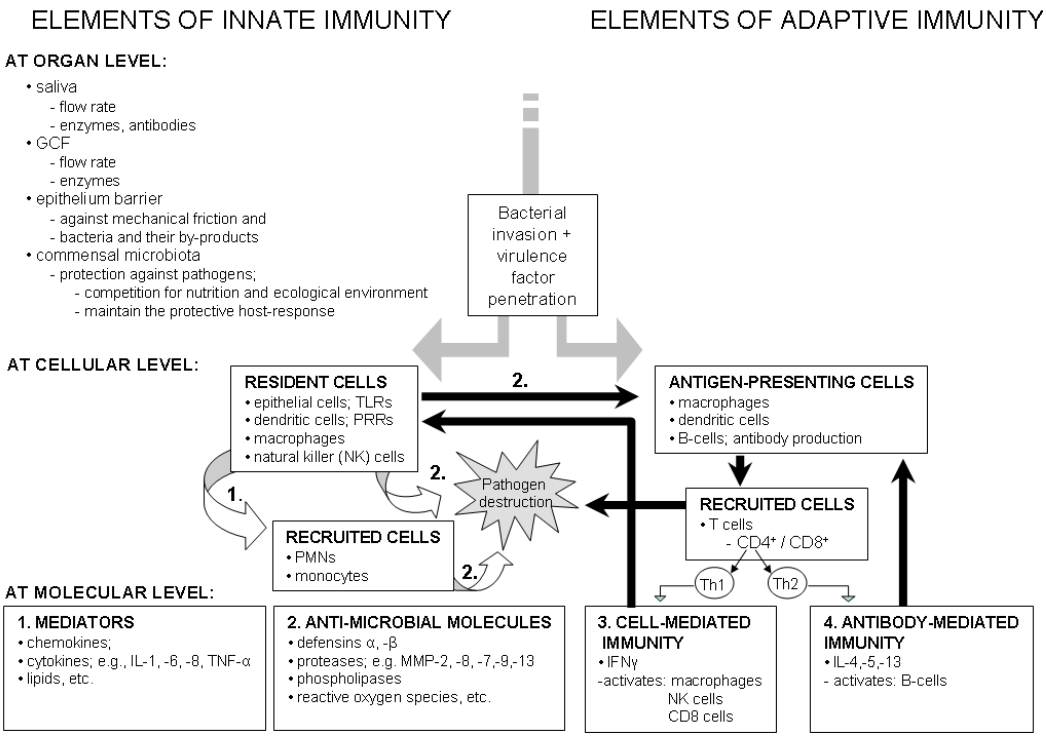


Figure 5. Schematic description of innate and adaptive immune responses. (Adapted from the text by Preshaw and Taylor, 2011).

Excessive degradation of the periodontal soft and hard tissues is mainly regulated by MMPs (Sorsa *et al*., 2006). MMPs are zinc-dependent endopeptidases, which can degrade extracellular matrix proteins at a neutral pH. Several resident and inflammatory cells of the periodontium, such as keratinocytes, fibroblasts, periodontal ligament cells, osteoblasts, osteoclasts, neutrophils, monocytes, macrophages, and plasma cells, produce different types of MMPs (Sorsa *et al*., 2004; 2006). Secretion and activation of MMPs are mainly regulated by bacterial products or by host cytokines, while their activation can be inhibited by tissue inhibitor of matrix metalloproteinases (TIMPs) (Sorsa *et al*., 2006). Predominant MMPs in the pathogenesis of periodontitis are neutrophil-derived MMP-8 (collagenase-2) and MMP-9 (gelatinase-B), which are present in GCF and saliva (Mäkelä *et al.*, 1994; Ingman *et al*., 1996). Among all the other MMPs, collagenases (MMP-1, -8, -13, and -18) degrade type I collagen, the main type of collagen in periodontium, in fibrillar form. Gelatinases (MMP-2 and -9) contribute to the degradation of denatured collagen, and in addition, MMP-2 digests fibrillar collagen (Aimes and Quigley, 1995; Varani *et al*., 2002). In the pathogenesis of periodontal diseases, MMPs not only act as tissue-degrading enzymes, but can also form an activation cascade with each other, which can protect them from TIMP inhibition (Hernández Ríos *et al*., 2009).

Prolonged bacterial challenge, concomitantly with the progressive host response, allows the T- and B-lymphocytes to emerge at the sites of infection as an antigen-specific response (Preshaw and Taylor, 2011). As the main cells of adaptive immune system, T- lymphocytes regulate the cell-mediated immunity, while B-lymphocytes are mainly associated with the antibody-mediated immunity.

While the host defence mechanisms, mainly phagocytes and lymphocytes, try to suppress the level of infection by eliminating the pathogens, the increased expression of proinflammatory cytokines, such as IL-1β, -6, and TNF-α, stimulates the katabolic activity in the periodontal tissues (Garlet *et al.*, 2006; Trombone *et al*., 2009). In fact, these cytokines can induce the connective tissue and alveolar bone destruction, by increasing the expression of their own degradative mediators, such as Receptor Activator for Nuclear Factor κB Ligand (RANKL) and MMP-8 with a simultaneous down-regulation of their endogenous inhibitors, *i.e*. osteoprotegerin (OPG) and TIMP-1, respectively.

2.3.3 Modifying factors

The host's inflammatory response to infection is influenced by several environmental and systemic conditions, as well as by genetic factors, which, in the end affect the occurrence, progression, and severity of periodontal diseases (Stabholz *et al*., 2010). Environmental and behavioural factors, such as smoking and poor oral hygiene, may cause shifts in subgingival bacterial profiles (Haffajee and Socransky, 2001; Shchipkova *et al.*, 2010). Smoking itself also impairs the periodontal tissue homeostasis through its effects on wound healing and tissue regeneration. Tomar and Asma (2000) have reported that smokers have an up to four times higher risk of developing periodontitis in comparison to non-smokers, and the relationship between the number of cigarettes smoked daily and development of periodontitis is dose-dependent (OR 2.8 for ≤9 cigarettes per day and OR 5.9 for ≥31 cigarettes per day). Certain systemic diseases, such as diabetes, and neutropenia, impair the immune response and diminish tissue regeneration (Stabholz *et al*., 2010). Furthermore, genetic disorders and polymorphisms, such as in toll-like receptors and cytokine expressions and functioning, may alter bacteria recognition and host-signalling systems.

During different phases of women´s reproductive life, such as in puberty, menstrual cycle, pregnancy, and menopause (Amar and Chung, 1994; Mascarenhas *et al*., 2003), the bacteria-host tissue interaction is modified, either by enabling enhanced growth of certain bacteria, or by impairing the host-defence mechanisms.

2.4 FEMALE SEX STEROID HORMONES

Cholesterol-derived steroids form a large group of hormones, which can be additionally subgrouped into three main sets: corticosteroids, calcium-regulating steroids, and sex steroids, which include androgens, estrogens, and progestins (Mariotti, 1994; Mascarenhas *et al*., 2003).

In women, estrogens are responsible for essential reproductive functions such as development, growth, and maintenance of secondary female sex characteristics, *i.e.* mammary gland development (Mariotti, 1994; Mealey and Moritz, 2003). Estrogens appear naturally in three forms; estrone, estradiol, and estriol. Principally the ovarian follicles, and to a lesser extent the peripheral tissues, but also the placenta during pregnancy, secrete estradiol, whereas estrone is mainly secreted by the peripheral tissues and to a lesser extent by the ovaries. Estriol is predominantly a metabolite of estrone and estradiol. Estradiol is the most potent estrogen, and in fertile-aged women the most abundant one.

Progesterone is synthesised and secreted primarily in the ovaries by the corpus luteum during the latter half of the menstrual cycle, and in the placenta during pregnancy, but also by the adrenal cortex (Mariotti, 1994; Mealey and Moritz, 2003). The primary activities of progesterone are seen during the menstrual cycle and during pregnancy. Synergistically acting with estrogens, progesterone controls the menstrual cycle by regulating the gonadotropin secretion from the the anterior pituitary gland (Amar and Chung, 1994). During pregnancy, its main functions are to maintain the endometrial effect and the uterine quietence. Additionally, progesterone takes part in other body functions, such as decrease in hepatic secretion of very low density lipoprotein and high density lipoprotein, stimulation of the hypothalamic respiratory centre, enhancement of sodium excretion in kidneys and elevation of body temperature during ovulation (Mariotti, 1994).

Both estrogens and progesterone are found in very low (10^{-9}-10^{-12} mol/l) concentrations in the blood circulation, and the vast majority is bound to plasma proteins, *i.e.* albumin and globulins, such as the sex-hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) (Mariotti, 1994). Only the unbound (free) hormones are considered biologically active, and are able to enter the target cell by diffusion and bind to the specific hormone receptors and, then, initiate the hormone actions and effects in the involved tissue. Furthermore, unconjugated steroid hormones are able to enter the saliva by diffusing through the acinar and ductal cells of the salivary glands independently of the salivary flow rate (Vining *et al*., 1983). Therefore, the unconjugated salivary estrogens and progesterone concentrations closely reflect their unbound concentrations in plasma (Vining *et al*., 1983; Darne *et al*., 1987).

2.4.1 Estrogens and progesterone during pregnancy

From the fertilization of the ovum and implantation till the development of the placenta, the corpus luteum is responsible for the increased progesterone and estrogen production. The placenta takes over the role of the corpus luteum from the second trimester to term, and continues raising the female sex hormone production (Mariotti, 1994; Mealey and Moritz, 2003). The highest peaks of the progesterone and estrogen levels in serum are found in the end of the third trimester of pregnancy, when pregnant women daily produce approximately 20 mg of estradiol and almost 300 mg of progesterone (**Figure 6)**. When the placenta is disengaged at parturition, these female sex hormone levels remarkably decrease, reaching their non-pregnant concentrations within 2-3 days after delivery.

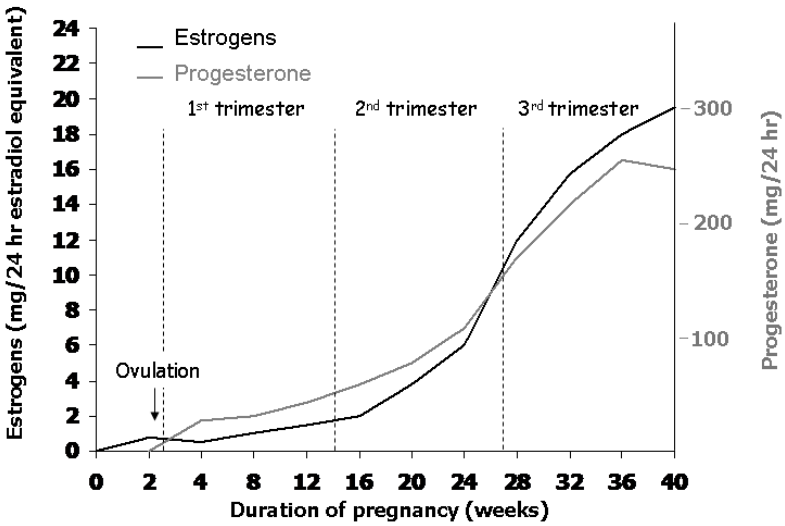


Figure 6. Rates of secretion of estrogens and progesterone at different stages of pregnancy. (Modified from Guyton, 1991).

During pregnancy, the key role of progesterone is to maintain the pregnancy (*i.e.* to allow the development of the fetus) by supporting the endometrium, decreasing the contractility of the smooth muscle in the uterus, and affecting the maternal immune response (Hansen, 1998; Szekeres-Bartho *et al*., 2001). Estrogens increase the size of the uterus, thicken the uterine wall and vaginal mucosa, and increase the blood supply by the enlarged number and size of vessels. Furthermore, together with estrogens, progesterone is needed for mammary gland development (Mariotti, 1994; Mealey and Moritz, 2003).

2.4.2 Steroid hormones and periodontium

It is well established that the regulation of cell proliferation, differentiation, and growth by estrogens and progesterone does not take place only in reproductive organs, but also occurs in other tissues. In fact, Vittek *et al*. (1982) reported that the gingiva represents a target tissue for estrogens by demonstrating for the first time a specific estrogen receptor (ER) in gingival tissue. Later, two distinct subtypes of receptors, ERα (Green *et al*., 1986; Greene *et al*., 1986) and ERβ (Kuiper *et al.*, 1996) have been found to mediate the direct effects of estrogens. Classical estrogen-target tissues, such as the endometrium, ovaries and mammary glands, contain predominantly ERα, whereas ERβ is also found in non-productive tissues, including periodontal ligaments (Jönsson, 2007; Mamalis *et al*., 2011), gingival epithelium (Nebel *et al*., 2011) and salivary glands (Välimaa *et al*., 2004). Besides ERβ, periodontal tissues also contain receptors for androgens (Parkar *et al*., 1996) and progesterone (Kawahara and Shimazu, 2003). This explains why periodontal tissues are sensitive to changes in circulating steroid hormone levels.

The effects of the female sex hormones on the periodontium are summarised in **Figure 7.** These are mainly based on observations concerning the biological changes that occur in

periodontal tissues during distinct hormonal conditions, such as puberty, oral contraceptive use, pregnancy, and menopause. Being a target tissue for steroid hormones, however, does not illuminate the complete etiology of sex-hormone-induced changes in the periodontium.

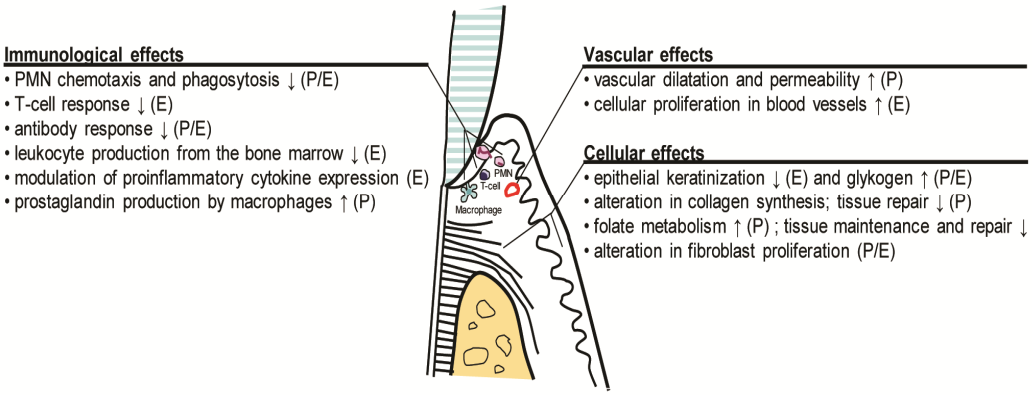


Figure 7. Summary of the estrogen (E) and progesterone (P) effects on the periodontium. (Modified from Amar and Chung, 1994; Mascarenhas *et al*., 2003)

2.5 PREGNANCY-RELATED PERIODONTAL CHANGES

Currently, pregnancy-associated periodontal changes, *i.e*. pregnancy gingivitis and pyogenic granuloma, are categorised under plaque-induced gingival diseases modified by systemic factors, here the endocrine system (Armitage, 1999). Pregnancy itself does not seemingly cause gingivitis, and a healthy gingiva without bacterial challenge usually remains unaffected (Hugoson, 1971; Arafat, 1974). According to the previous literature, the prevalence of pregnancy gingivitis varies widely between 35% (Hasson, 1960; Chaikin, 1977) and 100% (Löe and Silness, 1963; Hugoson, 1971), whereas the pregnancy-related pyogenic granuloma (epulis gravidarum) develops only in up to 5% of pregnant women (Tiililä, 1962). Independent of the study subjects' ethnicity, similar changes in the gingival appearance during pregnancy have been reported among different populations (Hugoson, 1971; Malisa *et al.*, 1993; Muramatsu and Takaesu, 1994; Tilakaratne *et al*., 2000; Figuero *et al*., 2010). On the other hand, Afro-American women (Lieff *et al.*, 2004) and women with poor socio-economic status have a significantly increased tendency to develop aggravated pregnancy-related periodontal changes (Machuca *et al*., 1999; Yalcin *et al*., 2002b; Taani *et al*., 2003; Sarlati *et al*., 2004).

In order to explain the etiology of pregnancy-related gingivitis, several potential mechanisms have been proposed, including effects of hormones, subgingival microbiota, and/or immune-inflammatory host response on periodontal tissues. However, so far the underlying mechanisms have not been completely clarified. **Table 2** presents the majority of existing data on pregnancy gingivitis from the previous longitudinal and prospective cohort studies. Although during the last 10-year period an increased number of case-controlled follow-up surveys have been published, most of the evidence related to pregnancy gingivitis is still available from cross-sectional and *in vitro* studies. This makes it difficult to obtain an exact association between

periodontal changes and pregnancy. Furthermore, the data comparison is very complex because of the vast disparity in the study materials and methods used in different surveys.

2.5.1 Clinical features

During pregnancy, there is an increase in gingival inflammation in the presence of stable (Silness and Löe, 1964; Kornman and Loesche, 1980; Tilakaratne *et al*., 2000, Figuero *et al*., 2010) or even decreasing plaque levels (Hugoson, 1971; O'Neil, 1979a). Clinically, gingival changes represent typical signs of inflammation, such as redness in colour, tissue swelling, increased tendency and severity of bleeding (Samant *et al*., 1976), and enhanced GCF secretion (Jensen *et al*., 1981). Besides those, gingival pocket formation (Löe and Silness, 1963; Hugoson, 1971; Muramatsu and Takaesu, 1994; Raber-Durlacher *et al.*, 1994; Yalcin *et al.*, 2002b; Taani *et al*., 2003; Buduneli *et al*., 2010) and enhanced tooth mobility (Cohen *et al*., 1971) have been detected. Inflammatory changes often occur in the interproximal sites and, usually, are significantly greater in molars than in anterior teeth (Löe and Silness, 1963; Hugoson, 1971; Moss *et al*., 2007). Interestingly, women with partly or fully erupted third molars are found to be at potentially increased risk of developing more severe periodontal inflammation during pregnancy in comparison with subjects without third molars (Moss *et al*., 2007).

Pregnancy-associated gingival inflammation reaches its highest peak during the second (O'Neil, 1979a; Kornman and Loesche, 1980; Muramatsu and Takaesu, 1994) or the third trimester (Löe and Silness, 1963; Cohen *et al.*, 1969; Hugoson, 1971; Tilakaratne *et al*., 2000; Yalcin *et al*., 2002a). It subsequently recovers spontaneously and, after delivery, the gingival health status is similar to that in non-pregnant women, without any apparent progress to periodontitis within a three-month (Cohen *et al*., 1969; Tilakaratne *et al*., 2000) up to a 15-month post-partum follow-up (Cohen *et al*., 1971).

Previously, clinical attachment level (CAL) during pregnancy has been evaluated only in few studies and the results vary. In an experimental gingivitis model (Raber-Durlacher *et al.*, 1994) and in a cross-sectional clinical study (Taani *et al*., 2003), no differences in CAL were found, whereas in previous longitudinal studies, no differences in CAL were found between pregnant and non-pregnant subjects (Tilakaratne *et al*., 2000) or some attachment loss was detected in pregnant women during the follow-up (Cohen *et al*., 1969). When any other modifying factor, such as diabetes (Guthmiller *et al*., 2001; Ruiz *et al*., 2011), race, or poor socio-economic status (Lieff *et al*., 2004) was present, the risk of having attachment loss and pocket formation increased significantly. Based on these varying results, it seems that there is still need for more information about whether prolonged and aggravated gingivitis during pregnancy is able to proceed to initial periodontal destruction, including increased pocket formation and attachment loss.

Another type of tissue reaction as an outcome of continuous low-grade bacterial irritation (typically involved with subgingival calculus or other plaque retention) during pregnancy is epulis gravidarum (Ojanotko-Harri *et al*., 1991; Jafarzadeh *et al*., 2006). This highly vascularised and easily bleeding gingival lesion on the interdental papilla usually exists as an exophytic mass with a sessile or pedunculated base (Tiililä, 1962; Ojanotko-Harri *et al*., 1991). The colour varies from dark red to purplish red or even blue, and the surface is often coated with small fibrin spots.

Table 2. Summary of the previous longitudinal cohort studies on pregnancy gingivitis

Study	Characteristics of subjects	Visits; Pr / N-Pr	Measurements	Main findings	Comments / additional findings
Cohen *et al*., 1969 (USA)	Pr; n=16 (24.6 ± 4.72 yrs)	Pr: 1st , 2nd, 3rd trimesters of pregnancy, and 3 months after delivery	GI a2, periodontal destruction a1, soft a2 & hard deposits a1, tooth mobility c	Plaque levels steady throughout the follow-up, during the 2nd trimester gingival inflammation ↑ and tooth mobility ↑.	Correlation between plaque and gingival inflammation scores at each visit except 2nd trimester.
Cohen *et al*., 1971 (USA)	Pr; n=16 (24.6 ± 4.72 yrs), 1 drop-out N-Pr: n=16 (age-matched)	Pr: 1st, 2nd, 3rd trimesters of pregnancy, and 3 and 15 months post-partum N-Pr: 0, 12, 24 months	GI a2, periodontal destruction a1, soft a2 & hard deposits a1, tooth mobility c	Gingival inflammation ↑ among Pr women in comparison to N-Pr women. Post-partum no differences between two groups.	
Hugoson, 1971 (Sweden)	Pr; n=26: 19-30 yrs	Pr: 12th, 18th, 24th, 30th, 34th, 38th wk of pregnancy, and post-partum: 1-3 days, 8 wks, 20 wks	PII c2, GI c2, PD c2. Additionally, gingival exudate was collected.	The lowest PII ($p<0.05$) and the highest GI scores ($p<0.001$) at 38th wks of pregnancy. The GI, PII, and PD higher in molars and approximal sites. GCF secretion ↑ during pregnancy.	No correlation between PII and GI. 11 women received scaling and oral hygiene instructions during the follow-up, 15 not before the final examination.
O´Neil, 1979a (England)	Pr; n=30: 18-32 yrs, 6 drop-outs N-Pr; n=30 (age -matched), 4 drop-outs	Pr: 14th and 30th wks of pregnancy, and 8 wks post-partum N-Pr: 2 visits 28 days apart near to the middle of the menstrual cycle	GI c2, PII c2. Additionally, venous blood was collected: peripheral blood lymphocytes tested for antigenic stimulation.	Between 14th and 30th wks of pregnancy: Plaque scores ↓, gingival inflammation ↑, maternal T-lymphocyte responsiveness ↓.	Most of the Pr and N-Pr subjects received scaling therapy and oral hygiene instructions between the visits.
O´Neil, 1979b (England)	Pr; n=30: 18-32 yrs, 6 drop-outs N-Pr; n=30 (age matched), 4 drop-outs	Pr: 14th and 30th wks of pregnancy, and 8 wks post-partum N-Pr: 2 visits 28 days apart near to the middle of the menstrual cycle	GI c2, PII c2. Additionally, venous blood was collected: plasma levels of estradiol and progesterone were measured.	Clinical findings same as above. A marked rise in estrogen and progesterone levels in plasma throughout pregnancy.	No correlation between clinical findings and hormone levels.

GI = gingival index; N-Pr = non-pregnant control population; PD = pocket depth; PII = plaque index; Pr = pregnant study population
Measured from: a all teeth, b all teeth except third molars, or c certain index teeth. The number of sites measured per tooth: 1 1 , 2 2-4 or 3 6

Table 2. Continues

Study	Characteristics of subjects	Visits; Pr / N-Pr	Measurements	Main findings	Comments / additional findings
Kornman and Loesche, 1980 (USA)	Pr; n=20, 20-33 yrs N-Pr; n=11, 22-36 yrs	Pr; monthly until delivery (at ≤13, 13-16,17-20, 21-24, 25-28, 29-32, 33-36, 37-40 wks of pregnancy), post-partum N-Pr; 4 visits, once per subsequent month	PII [C1], GI [C1], and BOP from interproximal sites mesial to first molars (max. 22 sites) . Subgingival plaque samples collected from 2 sites. Culturing on selective and non-selective media.	During the 2nd trimester: BOP ↑, (highest peak at 21-24 wks) and GI ↑. No significant changes in PII within and between the groups during the follow-up.	During the 2nd (21-24 wks) trimester: anaerobe/aerobe ratio ↑, *P. intermedia*; here "*P. intermedia* sensu lato" (% of total CFU) ↑ (p<0.01)
Tilakaratne *et al*., 2000 (Sri Lanka)	Pr; n=47, 17-36 yrs N-Pr; n=47, 17-36 yrs, matched controls	Pr; 1st , 2nd, 3rd trimesters of pregnancy, and 3 months after delivery N-Pr: 4 visits, one every 3 months	PII [a3], GI [a3], loss of CAL [b2]	Significant increases in GI during the follow-up being highest at 3rd trimester. The PI scores remained steady. No changes in CAL.	
Yalcin *et al*., 2002b (Turkey)	Pr; n=61, 17-36 yrs	Pr; 1st , 2nd, 3rd trimesters of pregnancy	PII [b2], GI [b2], PD [b2]	Significant increases in PI, GI, and PD scores during the follow-up, the highest scores were at 3rd trimester.	Women with lower educational level and with no history of previous periodontal care, had significantly higher PI, GI, and PD scores.
Yalcin *et al*., 2002a (Turkey)	Pr; n=22, 23-36 yrs	Pr; 1st , 2nd, 3rd trimesters of pregnancy	PII [a3], GI [a3], PD [a3]. PGE_2 levels in GCF samples collected from two anterior teeth.	Periodontal therapy reduced significantly the PGE_2 levels in GCF among PII, GI and PD values visit by visit during pregnancy.	Subjects received scaling therapy and oral hygiene instructions between the visits.
Lieff , 2004 (USA)	Pr; n=1067, 14-46 yrs, 85% (n=903) completed both visits	Pr; <26 wks of pregnancy, and within 48 hrs of delivery	PII [a1], GI [a2], BOP [a3], PD [a3], CAL [a3].	No significant changes in GI and BOP between two visits. However, a significant increase in a subgroup of women with ≥4 sites with ≥2 mm attachment loss.	A significant association between maternal periodontal disease status and ethnicity (African American), smoking, and insurance status.
Moss *et al*., 2005 (USA)	Pr; n=891, 14-46 yrs	Pr; <26 wks of pregnancy, and within 48-72 hrs of delivery	PII [a1], GI [a1], BOP [a3], PD [a3], gingival recession [a3]. Both person-based and site-based analyses were performed to analyse disease incidence and progression.	Increased PD concomitant with BOP during pregnancy may predict gingivitis/ periodontitis insidence/ progression.	1.7% of sites occurred PD ≥4 mm during pregnancy. Among those sites, 98 % was non-diseased at baseline examination.

BOP = bleeding on probing; CAL = clinical attachment level; CFU = colony forming unit; GCF = gingival crevicular fluid; GI = gingival index; N-Pr = non-pregnant control population; PD = pocket depth; PGE_2 = prostaglandin E_2; PII = plaque index; Pr = pregnant study population
Measured from: [a] all teeth, [b] all teeth except third molars, or [c] certain index teeth. The number of sites measured per tooth: [1] 1 , [2] 2-4 or [3] 6

Study	Characteristics of subjects	Visits; Pr / N-Pr	Measurements	Main findings	Comments / additional findings
Adriaens *et al.*, 2009 (Switzerland)	Pr; n=20, 26-42 yrs	Pr; 12, 28, and 36 wks of pregnancy, and 4-6 wks after delivery	BOP [a3], PD [a3], and gingival recession [a3] during the first and last visits. BOP [c1] and subgingival plaque samples from MB sites of first molars in all visits.	Spontaneously between wk 12 and post-partum: BOP↓ (p<0.01), the proportions of 17 (out of 37 detected) species ↓.	*P. gingivalis* and *T. forsythia* associated with BOP during the 1st trimester. However, their total counts remained stable.
Buduneli *et al.*, 2010 (Turkey)	Pr; n=43, 18-35 yrs	Pr; 2nd trimester of pregnancy, and 6-8 wks after delivery	VPI [a3]. BOP [a3], gingival recession [a3], and PD [a3]. t-PA and PAI-2 measured from pooled GCF samples , which were collected from 4 inflamed interproximal sites (single rooted teeth).	No difference in VPI and BOP between the visits. After delivery, the mean no. of pockets with PD ≥4 mm ↓. No significant changes in t-PA and PAI-2 levels.	5 of 43 women were smokers, and 11 of 43 former smokers. Oral hygiene level was poor, as VPI and BOP scores were very high at both visits.
Carrillo-de-Albornoz *et al.*, 2012 (Spain)	Pr; n=42, 16 drop-outs, 20–35 yrs N-Pr; n=20, 22-26 yrs	Pr; 12-14, 23-25, 33-36 wks of pregnancy, 3 months post-partum N-Pr: 2 visits with a 6-months interval	PII [a2], GI [a2], PD [a2]. Levels of IL-1β and TNF-α (d.13) and IL-6 and PGE_2 (d.23) in GCF. Pooled subgingival samples from 4 most inflamed sites. Estradiol and progesterone levels in unstimulated saliva.	Stable PI levels. GI ↑ significantly during the 2nd trimester, and remained at the same level until post-partum. During pregnancy, no changes in the proportions of the subgingival periodontal pathogens or IL-1β and PGE_2 levels, while IL-6 gradually ↑ and TNF-α ↓.	No. of PGE_2 samples < subjects in the Pr-group during pregnancy (n=23/42) and post-partum (n=14/26), and in the N-Pr-group (n=10/20).
Bieri *et al.*, 2012 (Switzerland)	Pr; n=19, 26-42 yrs	Pr; 12 wks of pregnancy and 4-6 wks after delivery	MB sites of 1st molars: IL-1α,-1β, -8, TNF-α, SLPI in GCF. Microbial analyses in subgingival plaque and clinical measurements are described previously by Adriaens *et al.* (2009).	Between wk 12 and post-partum: no difference in IL-1α,-1β, -8, TNF-α , and SLPI levels.	mRNA expression levels of cytokines do not associate with BOP, but correlate with each other.

BOP = bleeding on probing; GCF = gingival crevicular fluid; GI = gingival index; IL = interleukin; MB = mesiobuccal; N-Pr = non-pregnant control population; PAI-2 = plasminogen activator inhibitor-2; PD = pocket depth; PGE_2 = prostaglandin E_2; PII = plaque index; Pr = pregnant study population; SLPI = secretory leukocyte protease inhibitor ; TNF-α = tumor necrosis factor-alpha; t-PA = tissue type plasminogen activator ; VPI = visible plaque index
Measured from: [a] all teeth, [b] all teeth except third molars, or [c] certain index teeth. The number of sites measured per tooth: [1] 1, [2] 2-4 or [3] 6

2.5.2 Microbial aspects

Steady plaque levels concomitantly with a high increase in gingival bleeding during pregnancy have led to a proposal that the elevated levels of female sex hormones may also influence the composition and virulence of subgingival biofilms. In fact, along with the elevated levels of estrogens and progesterone in serum during pregnancy, qualitative changes in the subgingival microbiota from the aerobic or facultative gram-positive species towards anaerobic gram-negative species have been observed (Kornman and Loesche, 1980; Jensen *et al*., 1981). Especially during the second trimester, when gingival bleeding reaches its highest levels, the proportion of *P. intermedia* (formerly *Bacteroides intermedius*) in subgingival plaque has been reported to increase significantly (Kornman and Loesche, 1980; Muramatsu and Takaesu, 1994; Raber-Durlacher *et al*., 1994). Later, Kornman and Loesche (1982) explained this phenomenon by showing that two pigmented *Prevotella* species, *Prevotella melaninogenica* and *P. intermedia*, are actually able to use both estrogen and progesterone for their growth instead of vitamin K, which is an essential growth factor. A decade later, *P. intermedia* was found to contain two phenotypically indistinguishable species, *P. intermedia* and *P. nigrescens* (Shah and Gharbia, 1992). Thereafter, the term "*P. intermedia* sensu lato" has been proposed, to be used if these two species are not separated from each other. Nowadays the separation, however, can be performed reliably and without difficulties by using molecular biology methods, such as 16S ribosomal deoxyribonucleic acid (rDNA)-based polymerase chain reaction (PCR) techniques (Paster *et al*., 1994; Haraldsson and Holbrook, 1999). Since it is currently possible to identify *P. intermedia* and *P. nigrescens*, their association with pregnancy gingivitis remains to be individually clarified.

Within recent years, further clinical follow-up studies have been performed, aiming to evaluate shifts in the microbiological status during pregnancy, to find any involvement of specific pathogens with pregnancy gingivitis, and to assess any correlations between the oral microbiota and elevated hormone levels during pregnancy. A few examples are given as follows:

Yokoyama and co-workers (2005) showed in their *in vitro* studies that estradiol is able to increase the growth of *C. rectus*, which is another potential periodontal pathogen. In their cross-sectional study, a positive correlation was found between the salivary estradiol concentrations and levels of *C. rectus, P. gingivalis,* and *F. nucleatum* in pregnant women (Yokoyama *et al.*, 2008). Additionally, the salivary *C. rectus* levels positively correlated with the percentages of sites with 4 mm pocket depth without any attachment loss.

According to a pilot study, when the third molars were present, the efficacy of scaling and root planing during pregnancy proved to be limited to reduce the amount of periodontal pathogens measured from the mesiobuccal sites of first molars (Moss *et al*., 2008). Especially, increased counts of *T. forsythia* and *P. nigrescens* were significantly associated with the presence of third molars. Thus, third molars in pregnant subjects are suggested to act as a reservoir for periodontal pathogens by serving as suitable niches for their growth.

However, there are discrepancies in the current literature about the correlation between increased hormone levels and subgingival microbiota during pregnancy. In a follow-up

study, no significant changes in the proportions of subgingival *P. intermedia* (here named as *P. intermedia* sensu lato) were found between pregnant and non-pregnant subjects (Jonsson *et al*., 1988). In addition, no correlation was found between the microbiological or clinical parameters and hormonal status. In contrast, in a recent study by Carrillo-de-Albornoz *et al*., (2010), pregnant women without periodontitis, harbouring *P. gingivalis* or *P. intermedia* (here named as *P. intermedia* sensu lato) in subgingival biofilms, presented a significantly increased gingival inflammation tendency during mid-pregnancy and the presence of *P. intermedia sensu lato* and *P. gingivalis* positively correlated with the salivary female sex hormone levels.

Furthermore, interesting insights are provided in a recent study, where the occurrence of two subgingival herpes viruses, *i.e.* Epstein-Barr and cytomegalovirus, was examined at 28-32 weeks of pregnancy (Ereş *et al.,* 2011). The intra-group analysis indicated that the virus occurrence in relation to the sites with pregnancy gingivitis did not significantly differ from those with the healthy condition. However, the presence of Epstein–Barr virus was over three-fold ($p<0.05$) in pregnant women in comparison with non-pregnant controls.

Based on these results, the effects of female sex hormones on subgingival microbiota remain generally conflicting, as the results from different studies are still inconclusive, and, thus, no clear etiology for pregnancy gingivitis can be given.

2.5.3 Gestures in host response

Elevated progesterone levels aim to allow the fetal survival throughout pregnancy (Hansen, 1998), by, for example, suppressing the mother's own immune response against the fetus. In other words, immunosuppression during pregnancy is of benefit to the fetus; however, the remodulation of the immune response also affects the mother's host-defence against bacterial challenge (Szekeres-Bartho *et al*., 2001; Chen *et al*., 2012). An increased susceptibility to gingival inflammation during pregnancy might, at least partly, be explained by the sex-hormone-related immunosuppression.

It has been demonstrated that high progestrone levels have effects on local inflammation by down-regulating the IL-6 production of gingival fibroblasts (Lapp *et al*., 1995). Interestingly, these results were later challenged by Yokoyama *et al.* (2005), who observed an increase in the IL-6 and IL-8 production by estradiol-stimulated gingival fibroblasts (Yokoyama *et al.*, 2005). IL-6 is of major importance in the periodontal disease pathogenesis, as it stimulates the B- and T-lymphocyte differentiation, activates macrophages, and regulates osteoclastogenesis (Bouman *et al*., 2005). Therefore, its up- or down-regulation by estrogen modulates the disease and healing processes of periodontal tissues.

Collagen-degrading MMP productions of fibroblasts have been studied in *in vitro* conditions. When incubated with progesterone, IL-1β-stimulated MMP-1, -3, -8, and -9 secretions were down-regulated (Lapp *et al*., 2003). Overexpression of MMPs is of interest, since the main organic content of connective tissue and bone is collagen, which MMPs can degrade. However, our knowledge of the role of female sex hormones on MMPs and their regulators

in periodontal tissues during pregnancy is limited, and especially neutrophilic enzymes, including MMPs, need to be studied thoroughly.

In response to the challenge by pathogenic periodontal bacteria, gingival tissues respond to inflammation by up-regulating endothelial adhesion molecules, increasing the secretion of chemotactic agents, and aggravating leukocyte chemotaxis (Scott and Krauss, 2012). Estrogen attenuates these critical steps of inflammation by inhibiting the secretion of adhesion molecules and chemokines, *i.e*. macrophage chemoattractant peptide-1 and IL-8, respectively (Rodriguez *et al.,* 2002; Shu *et al.,* 2008). It has been suggested that changes in the plasma concentrations of estradiol and progesterone not only affect the chemotaxis and phagocytosis of PMNs (Björksten *et al*., 1978; Persellin and Thoi, 1979; Krause *et al*., 1987; Ito *et al*., 1995), but also their migration, motility, and deformability (Miyagi *et al*., 1992). Consequently, in *in vitro* conditions, the chemotactic ability and migration of PMNs were enhanced by progesterone and reduced by estradiol, whereas no effects on monocytes were observed (Miyagi *et al*., 1992).

In addition, sex hormones may modulate gingival inflammation by impairing the monocyte functions. In response to incubation with estradiol, an enhanced or decreased production of prostaglandin E_2 (PGE_2) from monocytes, at least in *in vitro* conditions, has been shown (Miyagi *et al*., 1993). The modulation was dose-dependent, *i.e.* a high estradiol concentration increased the production, whereas a low concentration reduced it. In addition, monocytes contribute to the direct immune response by producing cytokines, such as IL-1β, TNF-α, IL-6, IL-12, and IL-18. In a clinical follow-up study, where GCF samples were analysed and compared between pregnancy and post-partum, no changes in IL-1β and TNF-α levels were found (Bieri *et al*., 2012). In contrast, according to Luppi *et al*. (2002), monocytes from pregnant women produce more IL-1β and IL-12, and less TNF-α, in comparison with the non-pregnant stage. Down-regulation of TNF-α was also seen in the T-lymphocyte response, together with an up-regulation of IL-4, IL-5, and IL-10. The suppression of cell-mediated immune response was explained by a shift from the Type 1 (Th1-mediated) to Type 2 (Th2-mediated) reactivity (Piccinni *et al*., 1995; Luppi, 2003). Interestingly, the function and antibody production of B-lymphocytes are unaffected in pregnancy (Brabin, 1985). These findings on systemic response do not correlate with an experimental gingivitis model during pregnancy; in that model, the number of B-cells decreased during pregnancy, but the number of Th1 cells increased (Raber-Durlacher *et al*., 1993). Overall, the role of T- and B-cells and their inner shifts in the pathogenesis of pregnancy-related periodontitis still need to be studied further.

3. AIMS OF THE STUDY

The etiology of pregnancy gingivitis most likely includes a multifactorial interaction between the periodontal tissues, female sex hormones, microbes, and host immune response **(Figure 8)**. Therefore, studies I-IV aimed to investigate longitudinally the effect of pregnancy on periodontal tissues, to evaluate different factors related to the development of pregnancy gingivitis, and to compare them with those in a matched group of non-pregnant women. The specific aims were:

- to examine the progression of pregnancy-related clinical changes in the periodontium during each trimester and post-partum (**I**)
- to determine the occurrence of *P. intermedia* and *P. nigrescens* during pregnancy and their involvement in pregnancy gingivitis (**II**)
- to examine the activation of host response during pregnancy **(III, IV)**
- to evaluate salivary **(III)** and GCF **(IV)** neutrophilic proteolytic enzyme involvements in pregnancy gingivitis

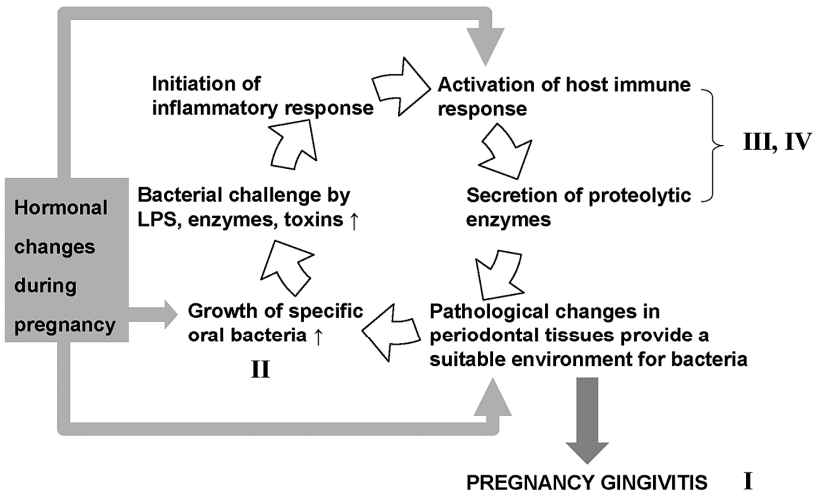


Figure 8. Proposal for the pathogenesis cascade of pregnancy gingivitis.

4. MATERIAL AND METHODS

Detailed descriptions of the materials and methods are provided in the original articles **I-IV**.

4.1 SUBJECTS, SELECTION CRITERIA, ETHICAL PERMISSION

4.1.1 Pregnant study population (Pr group)

Recruitment announcements, including details about the study with the selection criteria, were published in the local newspaper and also hung on the bulletin boards in the prenatal and dental clinics of the Kerava Municipal Health Care Centre. Furthermore, all the women, who attended their first check-up in the local prenatal clinic, were informed about the study. Of these, 30 generally healthy, non-smoking, pregnant Caucasian women (age range 24-35 years, mean 29.3 years), who were volunteers and fulfilled the selection criteria, were recruited for the study population (the Pr group).

4.1.2 Non-pregnant control population (N-Pr group)

Twenty-four matched non-pregnant Caucasian women (age range 25-36 years, mean 30.4 years) were recruited from the dental clinic of the Kerava Municipal Health Care Centre and enrolled as participants in the control population (the N-Pr group).

4.1.3 Inclusion / exclusion criteria

The selection criteria for the Pr and N-Pr groups at baseline are presented in **Table 3**.

Table 3. Inclusion and exclusion criteria for the participants in the study

	Pr group	N-Pr group
Inclusion criteria:		
❖ Women 24-36 years of age	●	●
❖ 10 ± 1 weeks of pregnancy *(for the Pr group only)*	●	
❖ Non-smoker or former smoker	●	●
❖ Presence of ≥20 natural teeth (besides third molars)	●	●
Exclusion criteria:		
❖ Pregnancy or breastfeeding *(for the N-Pr group only)*		●
❖ Present or previous diagnosis of periodontitis	●	●
❖ Presence of systemic disease/medication affecting the periodontium	●	●
❖ Systemic or topical antimicrobial or anti-inflammatory therapy within the previous 3 months	●	●
❖ Poor oral hygiene, deep caries lesions, remnant roots	●	●

4.1.4 Study design

The study protocol and sampling strategy are presented in **Figure 9**. This flow diagram also includes information on the derivation of the pregnant and non-pregnant women during the follow-up. The different time-points for each visit were set with slight modifications based on a corresponding longitudinal study scheme by Kornman and Loesche (1980).

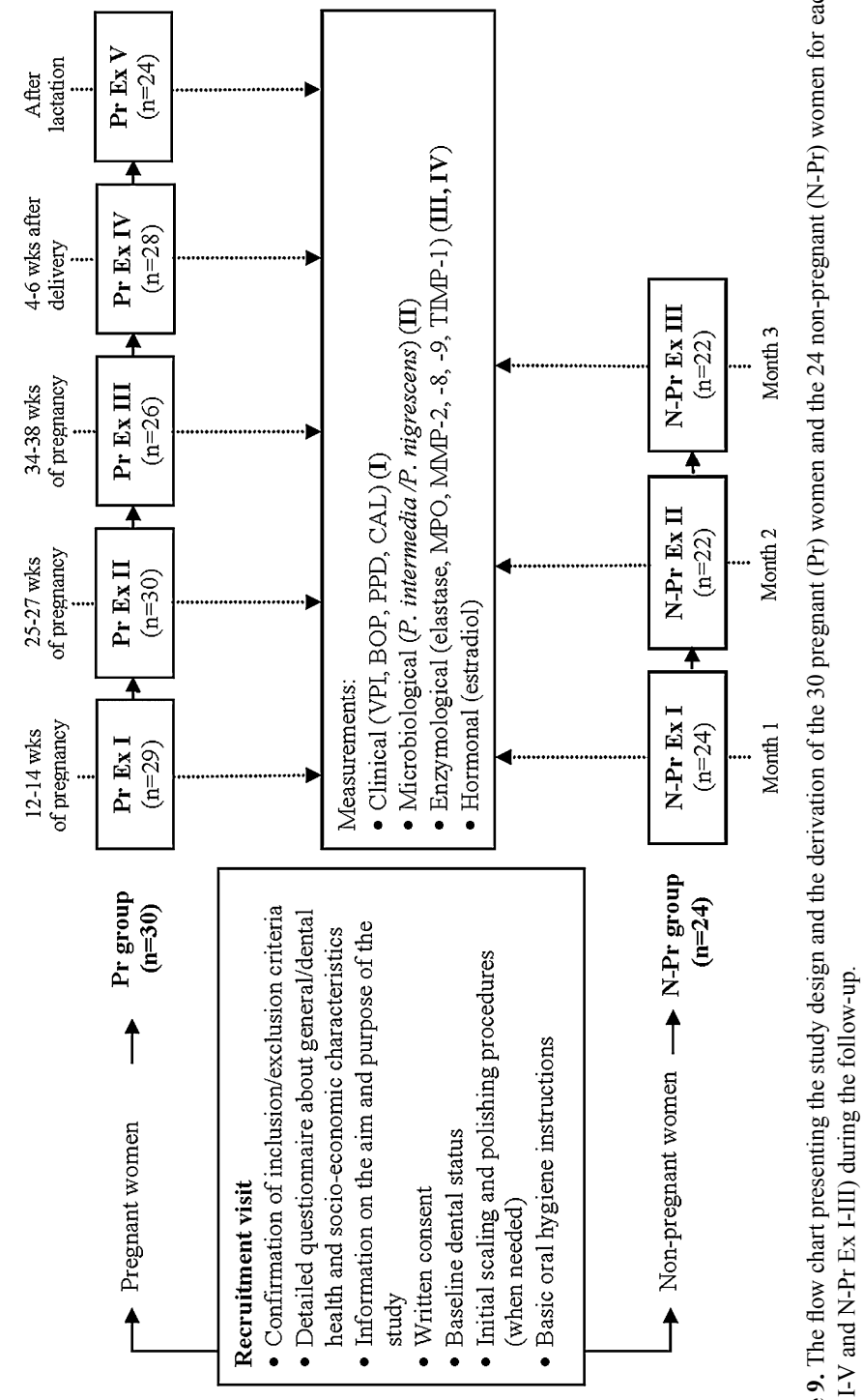


Figure 9. The flow chart presenting the study design and the derivation of the 30 pregnant (Pr) women and the 24 non-pregnant (N-Pr) women for each visit (Pr Ex I-V and N-Pr Ex I-III) during the follow-up.

4.1.5 Ethical permission

The study complied with the Declaration of Helsinki. Approvals for the study were obtained from the Helsinki University Central Hospital Obstetrics and Gynecology Ethics Committee and Kerava Municipal Health Care Centre. All participants in the study were informed on the purpose and objectives of the study, and their written consent was obtained.

4.2 CLINICAL EXAMINATION (I)

Clinical examinations were performed by one dentist (M.G.) between October 2002 and October 2006. After the initiation of the study, the intra-examiner agreement was tested by a calibration process including two periodontal examinations performed twice in the same subject (n=10) during the same day. Kappa scores were calculated for tested clinical parameters and the intra-examiner agreement was found to be very good (κ 0.853-0.897).

A summary of the clinical data collection for each study **(I-IV)** is presented in **Table 4**.

In studies **I** and **III**, at each visit, full-mouth clinical documentation of the periodontal status, including VPI, BOP, PPD, and CAL measurements, is presented from six sites per tooth.

In study **II**, full-mouth BOP recordings are presented as percentages.

In study **IV**, clinical measurements, including VPI, BOP, PPD, and CAL, are scored from the GCF sampling sites, the mesiobuccal sites of all first molars.

Table 4. Summary of clinical, microbial, enzyme, and other data in regard to their collection sources **(I-IV).**

	Sources of sample/data collection				
	6 sites/all teeth	**1 site/ quadrant*)**	**Subgingival plaque*)**	**Stimulated saliva**	**GCF*)**
Clinical data:					
BOP	**I, II, III**	**IV**			
CAL	**I, III**	**IV**			
PPD	**I, III**	**IV**			
VPI	**I, III**	**IV**			
Microbial data:					
P. intermedia / P. nigrescens			**II**	**II**	
Pigmented *Prevotella*			*unpublished*	*unpublished*	
Enzyme data:					
Elastase				**III**	**IV**
MMP-2				**III**	
MMP-8				**III**	**IV**
MMP-9				**III**	
MPO				**III**	**IV**
TIMP-1				**III**	**IV**
Other data:					
Estradiol				*Gürsoy et al.,2011*	
Salivary flow rate				*unpublished*	

***)** mesiobuccal sites of 1st molars

4.3 SPECIMEN COLLECTION

A summary of the sample and data collection for each study **(I-IV)** is presented in **Table 4.** At each visit, GCF, subgingival plaque, and stimulated saliva were collected. GCF and plaque samples were collected from the mesiobuccal sites of all first molars or, if missing, second molars.

4.3.1 Gingival crevicular fluid (IV)

Prior to collecting GCF, the sampling sites were gently dried with air and kept dry with cotton wool rolls, avoiding any contamination with saliva. For absorbing GCF, two filter-paper strips per sampling site were used. The tip of each strip was placed into the gingival sulcus for a standardised period of 30 seconds. The strips with blood contamination were discarded. The strips were placed into dry plastic Eppendorf tubes and kept at -20°C until further analysis.

4.3.2 Subgingival plaque (II)

After the tooth surfaces were gently dried with air and kept dry with cotton wool rolls, a pooled subgingival dental plaque sample was taken with a sterile Mini Gracey 11/12 curette (LM-Instruments Oy, Parainen, Finland). The plaque sample was placed into VMGA III transport medium (Dahlén *et al.*, 1993) in a vial with glass beads. Samples were transported within 2 h and cultivated immediately.

4.3.3 Saliva (II, III)

Paraffin-stimulated saliva was collected by expectoration for 5 min, and the sample was placed in a plastic Nunc Cryo Tube (Thermo Fisher Scientific, Roskilde, Denmark). Salivary samples for microbial testing were transported within 2 h and cultivated immediately **(II)**. Samples for salivary proteinase analyses were kept at -20°C until assayed **(III).**

4.4 BACTERIOLOGICAL METHODS

4.4.1 Cultivation, colony isolation, and preliminary identification of pigmented *Prevotella* species (II, additional data)

The saliva and subgingival plaque specimens were thoroughly vortexed, serially diluted (10^{-1} -10^{-5}), and cultured on non-selective Brucella agar for the total anaerobic growth and on selective kanamycin vancomycin laked blood (KVLB-2) agar for the detection of pigmented *Prevotella* species. The plates were incubated in anaerobic conditions at 37°C for 5-7 days until the isolation and subcultivation of potential pigmented *Prevotella* colonies on rabbit laked blood (RLB) agar, egg yolk agar (EYA), and Brucella agar; these were anaerobically incubated for 3-5 days. Whenever possible, at least 10 black-pigmented and 10 other pigmented isolates per specimen were collected from the KVLB-2 agar plates.

Biochemical reactions for a differentiation of pigmented *Prevotella* species are described in detail in **Table 5**. The preliminary identification was made according to the colony description, Gram staining (gram-negative short rods or coccobasilli), aerotolerance (negative; no growth in O_2 or CO_2), and phenotypic testing, including pigment production (tan/brown/dark brown/black colonies) on RLB agar, red fluorescence under ultraviolet light, lipase reaction (negative or positive) on EYA, reduction of nitrate (negative), and production of catalase (negative) and indole (negative or positive) (Jousimies-Somer *et al*., 2002).

Table 5. Biochemical reactions for a preliminary identification of different pigmented *Prevotella* species

	Gram staining	Pigment	Fluorescence	Lipase	Nitrate	Catalase	Indole	O_2	CO_2	Vancomycin	Kanamycin	Colistin	Penicillin
P. denticola	-	+	+	-	-	-	-	-	-	R	R	V	S
P.intermedia sensu lato	-	+	+	+	-	-	+	-	-	R	R^S	S	S
P. loescheii	-	+	+	-	-	-	-	-	-	R	R	V	S
P. melaninogenica	-	+	+	-	-	-	-	-	-	R	R	V	S
P. pallens	-	+	+	-	-	-	+	-	-	R	R^S	S	S

R= resistant, V= variable reaction, S= sensitive

4.4.2 DNA isolation for PCR (II)

Preliminarily identified *P. intermedia* sensu lato isolates from saliva (n=1,235) and subgingival plaque (n=1,393) were further identified by 16S rDNA-based PCR with slight modifications (Haraldsson and Holbrook, 1999). The details of the method used are given in **Study II**.

4.5 IMMUNOLOGICAL METHODS

Frozen saliva samples were thawed at room temperature and then centrifuged at 10,000 rpm for 3 minutes. The supernatants were aliquoted for further analyses **(III)**. According to Emingil *et al.* (2008), the absorbed GCF samples from each paper strip were eluted into 75 µl of 50 mM Tris-HCl (pH 7.8), including 0.2 M NaCl and 1 mM $CaCl_2$, on the shaker at room temperature for 2 h, and then stored at -20°C until enzyme analyses **(IV).**

4.5.1 Enzyme-linked immunosorbent assay (ELISA) (III, IV)

Levels of elastase, MPO, and TIMP-1 in saliva **(III)** and GCF samples **(IV)** were measured by commercially available ELISA kits, as recommended by the manufacturer. Estradiol levels in saliva (Gürsoy *et al*., 2011) were measured by the High Sensitivity Salivary 17β-Estradiol Enzyme Immunoassay Kit (Salimetrics, State College, PA, USA) according to the manufacturer's instructions. The amount of each salivary biomarker was analysed based on the concentration data **(III).** However, biomarkers in GCF are presented as total amount per standardised collection time of 30 s, thus avoiding the influence of inflammation on simultaneous effects on the biomarker and GCF increase (Lamster *et al.*, 1988; 1997; 2007).

4.5.2 Immunofluorometric assay (IFMA) (III, IV)

A time-resolved immunofluorometric assay (IFMA) with specific monoclonal antibodies, including catching antibody and europium-chelate labeled tracer antibody (Hemmilä *et al*., 1984), was used for detecting MMP-8 levels in saliva **(III)** and GCF samples **(IV)**, as described previously by Hanemaijer *et al*. (1997). The specificity of the monoclonal antibodies against MMP-8 corresponded to that of polyclonal MMP-8 (Sorsa *et al*., 2010).

4.6 FUNCTIONAL ASSAY

4.6.1 Gelatinase assay by zymography (III)

Gelatin zymography was used to identify the presence of two gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B) from saliva, based on their molecular weights 72 kD and 92 kD, respectively (Ingman *et al*., 1994; Sorsa *et al.*, 1997). The incubation of saliva specimens with the sample buffer was carried out at room temperature in the dark for 2 h. Then, 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels containing 1 mg/ml of fluorescent gelatin (Fluka, Buchs SG, Switzerland**)** as substrate were used (Sorsa *et al*., 1997). Under denaturing and non-reducing conditions, protein separation by electrophoresis was carried out at 4°C with constant voltage (110 V) for 1.5 h. After electrophoresis, partial protein renaturation and recovered enzymatic activity were reached by washing and incubating the gels first in 50 mM Tris-HCl (pH 7.5), including 0.02% (w/v) NaN_3 and 2.5% Tween 80 for 30 min, and then in the same buffer, supplemented with 5 µM $ZnCl_2$ and 1 mM $CaCl_2$ for 30 min. Finally, the gels were incubated at 37°C in a suitable activation buffer 50 mM Tris-HCl (pH 7.5), including 0.02% (w/v) NaN_3, 5 µM $ZnCl_2$, and 1 mM $CaCl_2$ for 18 h. After incubation, long wave UV light was used for visualizing the degraded gelatin, and then the gels were fixed and stained with 0.1% Coomassie Blue R-250 in 30% methanol and 10% acetic acid. The gelatinolytic levels and activities were visualised as clear bands against a blue background. Prestained low range SDS-PAGE standards (Bio-Rad, Hercules, CA, USA) were used to confirm the molecular weights of the gelatinolytic activities. The levels of MMP-2 and MMP-9 were determined using a scanning densitometry with Kodak molecular imaging software (Rochester, NY, USA), and expressed as relative levels derived from densitometric units (Sorsa *et al*., 1997).

4.7 STATISTICAL ANALYSES

The retrospective power calculation indicated that 26 women in each group would show a mean difference of 10% (SD 15%) on BOP index using a statistical power of 90% with a significance level of 5%. The calculation was performed with SAS Power and Sample Size 3.1 (SAS Institute INC Carry, NC, USA).

Data analyses were performed using the SPSS 14.0 **(I, II)** and 15.0 **(III, IV)** statistical softwares (SPSS Inc., Chicago, IL, USA). Analyses for each study parameter were carried out using the subject **(I-III)** or the site **(IV)** as the experimental unit. The Kolmogorov-Smirnov test and histograms were used to check the normality of the data distribution (Chan, 2003a). Because of the skewed nature of the data, all statistics were run through with non-parametric tests. Within the Pr and N-Pr groups, the differences between the follow-up visits were identified first with the Friedman test and then compared using the Wilcoxon signed ranks test (Chan, 2003b) **(I-III)**, and with the McNemar test (Chan, 2003c) **(IV)**. Furthermore, the Mann–Whitney U test was used for the comparison of the values between the Pr and N-Pr groups (Chan, 2003b) **(I-IV)**. Correlations between the clinical parameters and estrogen levels were calculated with the Spearman's rank correlation test (Chan, 2003d). In all comparisons p-values <0.05 were considered statistically significant.

5. RESULTS AND DISCUSSION

5.1 GENERAL AND METHODOLOGICAL ASPECTS

The recruitment of pregnant and non-pregnant women for the study was a demanding task. From autumn 2002 to autumn 2003, altogether 310 pregnant women visited the prenatal clinic, of whom 248 (80%) were potentially eligible participants by their age (24-35 years). The majority of them, however, did not meet the selection criteria or otherwise lacked interest in committing to a long follow-up study. Only 22 (8.9%) pregnant women contacted the principal investigator for further details about the study and made an appointment for the initial screening, but three of them were excluded because of the use of penicillin within a week, use of toothpaste with antimicrobial compound or a diverse time-schedule. Thus, 19 women were enrolled in the study during the first year. Therefore, the recruitment continued until 30 pregnant women were included in the study. Similarly, the lack of common interest was the main reason for even a smaller number of participants in the N-Pr group, *i.e*. during a four-year period, only 24 non-pregnant women were enrolled in the study.

The main strength of the present study series is its longitudinal study design with five scheduled follow-up visits for pregnant women, who were seen once per trimester and twice after delivery. In previous studies, the length of the post-partum follow-up period has varied widely from weeks (O´Neil, 1979a; Adriaens *et al*., 2009) to some months, typically up to three months (Cohen *et al*., 1969; Tilakaratne *et al*., 2000; Figuero *et al*., 2010), with the longest follow-up of 15 months (Cohen *et al*., 1971). In **studies I-IV**, the post-partum follow-up period for each subject was continued till the end of their lactation period, which ranged from 8 to 88 weeks with a mean duration of 38.7 ± 19.2 weeks. The final visit was arranged approximately 2-4 weeks after breastfeeding ended, however, not during menstrual bleeding. The current study is one of the longest ever undertaken.

The subjects in the Pr and N-Pr groups were recruited from one single health care centre, and they were homogenous in their ethnicity (Caucasian) and socio-economic status (**Table 6**). In previous pregnancy gingivitis studies, it has been shown that the socio-ecomic status and ethnicity may influence the clinical outcomes (Machuca *et al*., 1999; Yalcin *et al*., 2002b; Taani *et al*., 2003; Lieff *et al*., 2004). In addition, the age range of the present study population was limited to 24–36 years, since younger or older women may have hormonal fluctuations other than pregnancy, such as puberty or menopause (Amar and Chung, 1994; Mascarenhas *et al*., 2003). Also, the age of over 36 years has significant associations with increased PPD and loss of CAL in pregnant mothers (Machuca *et al.,* 1999). By limiting the age range, the potential influence of these variables on the periodontal condition of the present pregnant study population was avoided, thus increasing the strength of the results.

In the present study, any systemic disease, antimicrobial or anti-inflammatory drug use, which may have effects on periodontal findings, were excluded. In previous studies, pregnant women with diabetes were found to have a poorer periodontal condition in comparison with healthy pregnant women (Guthmiller *et al*., 2001; Ruiz *et al*., 2011). Smoking during pregnancy, on

the other hand, was found to have no significant effect on VPI, BOP, PPD, or composition of subgingival plaque (Buduneli *et al*., 2005). However, it is generally accepted that smoking affects the periodontium not only directly but also via microbial changes (Shchipkova *et al*., 2010) and altered host response (Mooney *et al*., 2001; Heikkinen *et al*., 2010). Therefore, smokers were excluded from the pregnant group in the current study. During pregnancy, the incidence or progression of periodontal inflammation has been shown to be more severe in periodontitis subjects than in periodontally healthy or gingivitis subjects (Moss *et al*., 2005). Therefore, also subjects with current or previous diagnosis of periodontitis were excluded from the present study.

Table 6. Demographic characteristics of the women in the pregnant (Pr) and non-pregnant (N-Pr) groups **(I)**

	Pr group; n=30		N-Pr group; n=24		*p*
Age (years)					
mean ± STD (min-max)	29.3 ± 2.8 (24-35)		30.4 ± 3.1 (25-36)		0.187
Marital status	**n**	**%**	**n**	**%**	
Single / divorced	2	7	2	8	0.227
Cohabitant	11	36	14	58	
Married	17	56	8	33	
Education level					
Basic	0	0	4	17	0.053
Secondary	18	60	10	42	
Higher	12	40	10	42	
Employment status					
Working full time	23	77	13	54	0.480
Working part time	1	3	2	8	
Student	2	7	2	8	
On child care leave	3	10	6	25	
Unemployed	1	3	1	4	
Number of teeth					
mean ± STD (min-max)	28.5 ± 1.4 (25-32)		28.6 ± 1.8 (24-32)		0.911

In **study I**, clinical parameters were measured from six sites per tooth, including third molars. Interesting insights into the role of third molars in the pathogenesis of periodontal disease have been provided by Moss *et al*. (2007, 2008). According to their findings, third molars can act as a reservoir for periodontal pathogens, hence, being the periodontal disease initiating region in the oral cavity. Despite this knowledge, third molars are often excluded from the clinical measurements, which may underestimate the clinical condition. Furthermore, in

studies II and IV, the microbial and enzyme collection was always performed from the same site in the same teeth. This is different from some studies, where subgingival plaque samples were collected from the most inflamed sites during each follow-up visit (Muramatsu and Takaesu, 1994; Carrillo-de-Albornoz *et al*., 2010) or where subgingival plaque and GCF samples were collected from different teeth (Figuero *et al*., 2010; Carrillo-de-Albornoz *et al*., 2010; 2012). However, such study designs would not allow the researcher to follow the effect of pregnancy on a specific site and, even less, to compare the level of host response with that of the microbial challenge. Therefore, the selection of the same site for collecting the samples at all follow-up visits strengthened the present study series.

Before the first visit (Pr Ex I or N-Pr Ex I), initial scaling and polishing procedures were provided at the recruitment examination in 18 of the 30 participants in the Pr group and in 13 of the 24 participants in the N-Pr group, who had slight supragingival calculus formation mainly on lingual sites of lower incisors, together with mild marginal gingival inflammation. Furthermore, all participants received initial oral hygiene instructions. The women were instructed to use only a dentifrice including standard fluoride formulation without any antimicrobial compound, thus avoiding potential errors in the results. According to the previous cross-sectional study, women who used a triclosan-containing dentifrice during pregnancy had a significant reduction in gingivitis development (Kraivaphan *et al.*, 2006). In a recent cohort study series, including clinical, microbiological, and immunological evaluations (Figuero *et al*., 2010; Carrillo-de-Albornoz *et al*., 2010; 2012), the pregnant and non-pregnant women were instructed to use a dentifrice with 0.30% triclosan, an antimicrobial compound, and 2% copolymer, which might have interfered with the study results. Based on a systematic review (Davies *et al*., 2004) and a meta-analysis (Hioe and van der Weijden, 2005), self-performed tooth brushing with a triclosan/copolymer-containing toothpaste improves the daily plaque control and reduces the gingivitis formation significantly. One reason for this is the diminished quantity and improved quality of subgingival microbiota after regular use of triclosan (Rosling *et al*., 1997). Additionally, a rather low concentration of triclosan can create an anti-inflammatory activity by inhibiting the IL-1β-induced PGE_2 production in gingival fibroblasts (Gaffar *et al*., 1995). Recently, triclosan has been found to suppress several other acute and chronic inflammatory mediators (Barros *et al*., 2010). On the other hand, a recent animal study demonstrated that triclosan enhances the overall estrogen effect on the body (Stoker *et al*., 2010). This, in turn, can, at least theoretically, affect the clinical outcome.

5.2 PERIODONTAL STATUS IN PREGNANT AND NON-PREGNANT WOMEN (I, IV)

Clinical observations in **studies I and IV** verify and further extend previous observations that periodontal tissues are more susceptible to inflammation during pregnancy. The subject-based changes in VPI, BOP and PPD scores of **study I** are presented in **Figure 10.** The highest scores of gingival bleeding ($p<0.01$) and pocket formation ($p<0.001$) occurred during the second trimester, which is in line with previous studies (O'Neil, 1979a; Kornman and Loesche, 1980; Muramatsu and Takaesu, 1994). Between the third trimester and 4-6

weeks after delivery, BOP and PPD scores decreased significantly ($p<0.001$). By the end of lactation, the scores reached the same levels as seen in non-pregnant women. VPI scores, on the other hand, decreased visit by visit during pregnancy and post-partum. A similar phenomenon occurring in two studies where study and control subjects received scaling and oral hygiene instructions between the visits, was explained by extended oral hygiene procedures (Hugoson, 1971; O'Neil, 1979a). However, this is not the explanatory reason in **study I**. In other studies, where the plaque levels were either steady or increased during pregnancy, gingival bleeding scores continued to increase after the second trimester, and reached their highest peak in the third trimester (Silness and Löe, 1964; Cohen *et al*., 1969; Hugoson, 1971; Tilakaratne *et al*., 2000; Figuero *et al*., 2010).

When 125 gingivitis patients were clinically examined, gingival bleeding was recorded by using quantitative and binary indices, *i.e.* GI and BOP measurements, respectively (Chaves *et al*., 1993). The highest correlation between these two parameters was found when PD was >2 mm. On the other hand, in the case of PD <2 mm, the greatest percentage (78%) of agreement was found when both parameters were absent. In **studies I** and **IV**, dental plaque and gingival bleeding were evaluated with binary indices (absence/presence). Although the use of quantitative indices has been preferred in most previous studies, their advantage is questionable in a follow-up study where clinical changes in pregnancy-related gingivitis are evaluated. It has been shown that the use of quantification indices may have little or no clinical benefit, while binary indices produce adequate data in monitoring the prevalence of clinical variables (Galgut, 1999). The validity of this statement was recently confirmed in a study by Figuero *et al*., (2010), where the clinical status of pregnant women was followed during pregnancy and post-partum. According to their results, mean gingival index values in the second trimester were 1.13 and post-partum 0.98, *i.e.* a decrease of 13%. The percentage of sites with positive bleeding (GI values of 1, 2, or 3 according to Löe and Sillness (1963)) was 58% in the second trimester and 50% post-partum, with a decrease of 13%. Therefore, the use of binary indices was accepted as a valid method for measuring gingival inflammation and possible disease progression also in the present study protocol.

Previous longitudinal follow-up studies have demonstrated that subjects with periodontitis and a mean BOP value of >20% have an increased incidence of periodontal disease progression (Lang *et al*., 1986; Joss *et al*., 1994), whereas the absence of gingival bleeding indicates periodontal stability (Lang *et al*., 1990). Furthermore, Schätzle and co-workers (2003) showed that a gingival site with repeatedly measured bleeding has an increased risk of proceeding to periodontitis. Based on these findings, **study I** included the aim to observe whether increased gingival bleeding during pregnancy is able to cause loss of attachment. The results demonstrated a high degree and severity of gingivitis, especially during the second and third trimesters, confirming the results of a previous cross-sectional study (Samant *et al*., 1976). In **study I**, the increased mean BOP values (≥30%) and the simultaneous increase in number of sites with PPD were associated during pregnancy. Furthermore, there was an association between decreased mean BOP values (≤20%) and diminished prevalence of deepened periodontal pockets post-partum. These results support the previous findings provided by Lang and Joss and their colleagues (1986; 1994), with the exception of a correlation between BOP and CAL, which was not observed in **study I**.

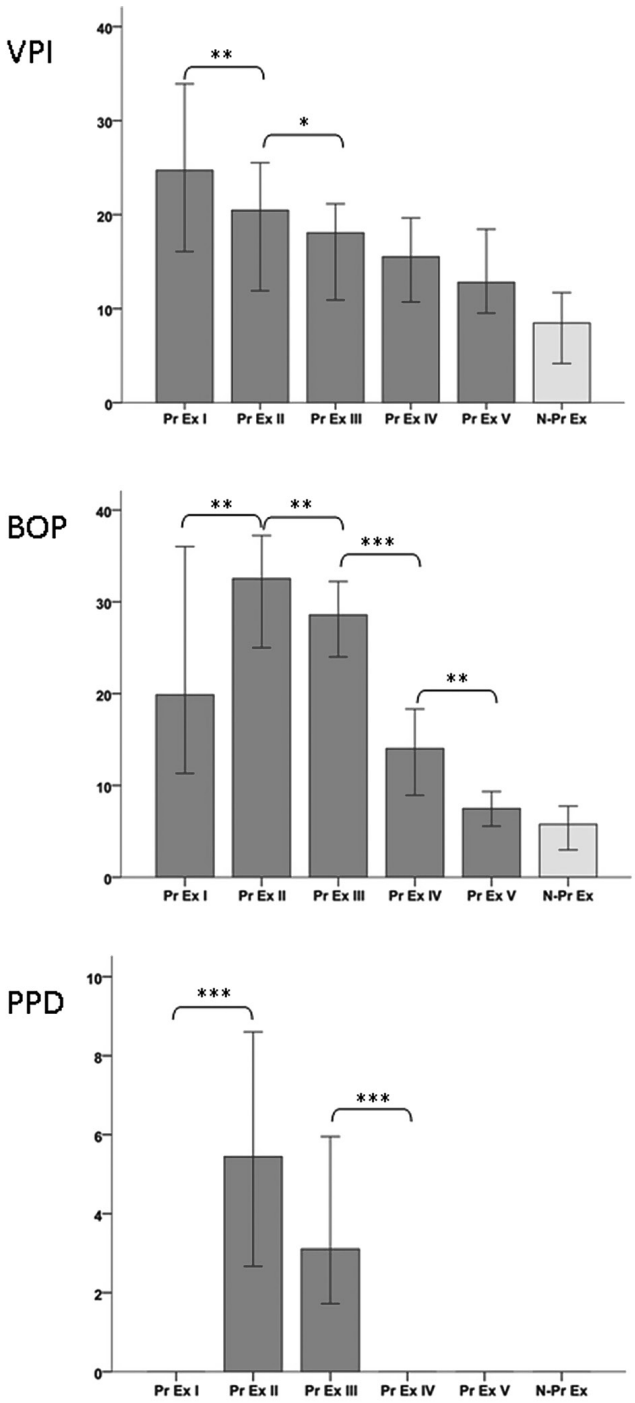


Figure 10. Percentages of visible plaque index (VPI) and bleeding on probing (BOP), and the number of gingival pockets with a probing pocket depth (PPD) ≥4 mm in pregnant women at five visits (Pr Ex I-III during the 1st, 2nd, and 3rd trimesters of pregnancy, respectively, Pr Ex IV after delivery, and Pr Ex V after lactation ended) and in non-pregnant women (N-Pr Ex). The results are given in medians with 95% Confidence intervals. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$

In the Pr group, ≥4 mm PPDs were mostly detected in interproximal sites and in posterior regions, which is in line with previous observations (Löe and Silness, 1963; Cohen *et al*., 1971; Moss *et al.*, 2007). The finding supports the proposal that the periodontal pocket formation during pregnancy is not caused by periodontal tissue breakdown, but occurs mainly due to gingival swelling. This is further confirmed by the unchanged CALs in pregnant women, being comparable with those in periodontally healthy non-pregnant women. Our findings are in line with previous studies where the pocket formation occurred as an outcome of increased gingival odema (Löe and Silness, 1963; Miyazaki *et al*., 1991; Buduneli *et al*., 2010) and CALs remained unchanged during pregnancy (Raber-Durlacher *et al*., 1994: Tilakaratne *et al*., 2000, Taani *et al*., 2003). Overall, not a single site in the Pr group showed characteristics to progress or transform to periodontitis during the follow-up. Therefore, according to the current results, prolonged gingival bleeding itself during pregnancy is not a risk factor for loss of attachment. However, to get more precise data, site-specific analyses are warranted. At present, there is only one study in the literature reporting the relation between deepened pockets with BOP at baseline and increased periodontal disease progression at a site-specific level (Moss *et al*., 2005). However, these findings need to be confirmed in other follow-up studies at site-specific level.

Subjects with type 1 or gestational diabetes during pregnancy have a significantly higher prevalence of periodontal inflammation and destruction measured as significantly higher BOP, PD, attachment loss, and tooth mobility scores in comparison to non-diabetic pregnant women (Guthmiller *et al*., 2001; Ruiz *et al*., 2011). Therefore, these conditions need to be evaluated separately.

5.2.1 Salivary estradiol levels and clinical findings (additional data)

A summary of the data collected from saliva is presented in **Table 7**. Paraffin-stimulated salivary flow rates remained stable during the follow-up, which is in line with a previous report by Laine *et al.* (1988), and the flow rate did not differ between pregnant and non-pregnant women. Salivary estradiol concentrations were measured during each visit. Those hormone levels increased significantly visit by visit throughout pregnancy and showed an immediate decrease ($p<0.001$) after delivery, reaching the same level as seen in non-pregnant women.

The accociation between VPI and salivary estradiol with BOP during the follow-up is presented in **Figure 11**. Despite profound alterations in gingival bleeding frequencies unrelated to overall amounts of visible plaque during pregnancy, VPI and BOP scores significantly correlated at all visits, whereas those of salivary estradiol were not related to BOP at any of the visits. Similar observations have been reported previously (O'Neil, 1979b; Muramatsu and Takaesu, 1984; Figuero *et al*., 2010). This finding may indicate an exaggerated inflammatory response by the host against relatively low amounts of plaque. In the N-Pr group, there was a high correlation between VPI and BOP scores, indicating a steady–state plaque environment.

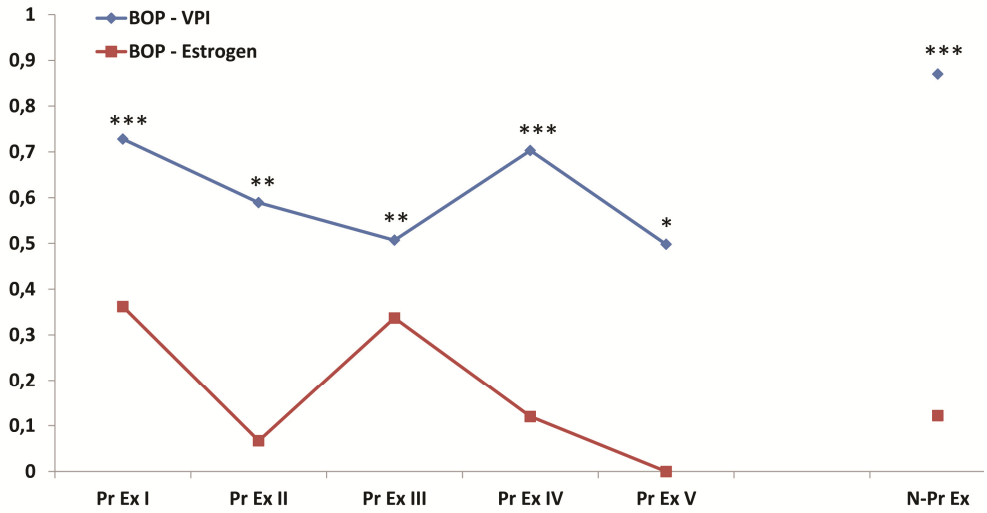


Figure 11. Correlations between visible plaque index (VPI) and salivary estradiol levels with bleeding on probing (BOP) scores in pregnant women at five visits (Pr Ex I-III during the 1st, 2nd, and 3rd trimesters of pregnancy, respectively, Pr Ex IV after delivery, and Pr Ex V after lactation ended) and in non-pregnant women (N-Pr Ex). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

5.3 MICROBIAL FINDINGS DURING PREGNANCY AND POST-PARTUM (II)

Gingival bleeding occurs as a result of the exposure to etiological periodontal bacteria (*i.e. A. actinomycetemcomitans, T. denticola, T. forsythia,* and *P. gingivalis*) in subgingival biofilms already in shallow gingival pockets with minimal attachment loss (Demmer *et al.*, 2008). On the other hand, certain bacterial species connected with periodontal health (such as *Actinomyces naeslundii* and *Veillonella parvula*) reveal an opposite association with BOP% and increased PD.

One aim of the present study was to determine the salivary and subgingival plaque levels of pigmented *Prevotella* species with culture-based methods. Initially, the culture-based method, using non-selective and selective media, was chosen, because bacterial cultures enable the selection and isolation of previously unknown species or strains. Additionally, the use of different isolates or strains for further studies is then possible. However, the chosen method also includes some limitations or weaknesses; it is vulnerable to methodological errors, it is time-consuming, and requires an extensive variety of materials. Although all pigmented *Prevotella*-like colonies were isolated, only *P. intermedia* and *P. nigrescens* were included in the current study **(II)**, while other strains were stored until further analyses.

Altogether 2628 isolates were preliminarily identified as *P. intermedia* sensu lato, and then further analysed and separated by a 16S rDNA-based PCR method. In the Pr group, 95.3% of the isolates proved to be *P. nigrescens* and 2.5% *P. intermedia*. In the N-Pr group, the corresponding findings were 94.2% and 5.5%. A small proportion of the isolates (<3%) remained unidentified. No explanation for this was found, since the system was successful in

identifying all reference strains of *P. intermedia* and *P. nigrescens*. Further studies are needed to characterise those isolates more precisely, keeping in mind the possibility of finding novel *Prevotella* species close to *P. intermedia* and *P. nigrescens*, as happened in a previous study describing *Prevotella pallens* (Könönen *et al*., 1998).

5.3.1 *Prevotella intermedia* sensu lato in subgingival plaque (II)

Kornman and Loesche (1980) found the highest anaerobe/aerobe ratio, *P. intermedia* (here "*P. intermedia* sensu lato") proportion in subgingival plaque, and gingival bleeding scores during the second trimester (at 21-24 weeks of pregnancy). Later, they demonstrated that "*P. intermedia* sensu lato" and *P. melaninogenica* (formerly *Bacteroides melaninogenicus*) are able to substitute estradiol and progesterone as their essential growth factor instead of vitamin K (Kornman and Loesche, 1982). However, the effect of female sex steroid hormones on bacterial growth is dose-dependent; the growth is stimulated within a very small and narrow concentration range and inhibited at higher concentrations. This supports the observation on the decreased proportions of *P. intermedia* sensu lato towards the end of the second trimester (at 25-28 wks of pregnancy) (Kornman and Loesche, 1980).

In **study II**, the second visit was scheduled at 25-27 weeks of pregnancy, when the highest peak of gingival bleeding was observed. In addition, the mean subgingival levels and proportions of *P. intermedia* sensu lato increased two-fold between the first and second trimesters. These increases, however, were not statistically significant. A possible reason for this finding could be, that the second visit was set up 3-4 weeks later than in the study of Kornman and Loesche (1980). According to Adriaens *et al*. (2009), plaque levels of *P. intermedia* and *P. nigrescens* were at their highest level in the end of the first trimester, and then decreased visit by visit during the follow-up. In their study, the second visit with a sample collection was performed at 28 weeks of pregnancy, at a time which can be affected by increased estrogen levels. However, during the second trimester, but not in the end of pregnancy or after delivery, *P. nigrescens* significantly increased ($p<0.001$) at sites presenting BOP.

In **study II**, a second increase ($p<0.05$) in the mean growth of *P. intermedia* sensu lato was found 4-6 weeks after delivery, in accordance with the report of Muramatsu and Takaesu (1994), where this finding was explained by advanced inflammation in periodontal tissues with prolonged healing. This explanation, however, is unlikely, since in **study I**, gingival inflammation reached its highest peak during the second trimester, after which all clinical parameters decreased visit by visit. As an alternative explanation, hormones may play a role in this specific phenomenon. In fact, Ojanotko-Harri *et al*. (1985) demonstrated that the tissue metabolism of progesterone varies depending on the clinical status of the gingiva. In comparison to a periodontally healthy condition, the progesterone metabolism is up to three-fold higher in inflamed gingiva, which leads to a very low amount of active hormone. On the contrary, during pregnancy, the progesterone metabolism is decreased, which consequently increases the amount of active hormone in the tissues (Ojanotko-Harri *et al*., 1991). This phenomenon leads to speculation about whether it also potentiates the hormonal effects in tissues during pregnancy. Moreover, although the hormonal concentrations in serum decrease immediately after the delivery, the amount of active hormone may remain longer in tissues.

This could explain the increase of *P. intermedia* sensu lato 4-6 weeks after delivery. Further studies are needed to prove this theory.

5.3.2 *Prevotella intermedia* sensu lato in saliva (II)

In **study II,** the mean growth and proportion of *P. intermedia* sensu lato remained rather stable in saliva during pregnancy (**Table 7**). Four-six weeks after delivery, the mean colony forming unit (CFU)/ml increased significantly, following the same trend seen in subgingival plaque. No such changes in subgingival or salivary levels were detected in non-pregnant women. Furthermore, the occurrence of salivary *P. intermedia* sensu lato was slightly lower than in subgingival plaque. This finding is in line with previous studies by Könönen *et al.* (1993; 1994). All women harboured *P. nigrescens*, either in saliva or in plaque, whereas the salivary carriage rate of *P. intermedia* varied between 17% and 21% in pregnant and non-pregnant women, respectively. Their carriage rates are similar to those studied previously in the Finnish population (Könönen *et al*., 2000; 2007)

Yokoyama *et al.* (2008) analysed saliva samples and detected certain periodontal pathogens by the PCR method. They found only an increase in the levels of *C. rectus*. They could not find any differences in *P. intermedia* levels during pregnancy or between pregnant and non-pregnant women. The reason for the contradictory results might be that they did not analyse *P. nigrescens* levels at all. Secondly, their cross-sectional study method did not allow them to take the stage of pregnancy into account, as they collected one sample per subject within various gestational ages, a range of 15 to 37 weeks. Korman and Loesche (1982) proved that estrogen improves the growth to some point, but at the end, as seen during the third trimester of pregnancy, high levels of estrogens suppress the growth of "*P. intermedia* sensu lato".

5.4 ALTERED HOST RESPONSE DURING PREGNANCY AND POST-PARTUM (III, IV, ADDITIONAL DATA)

The present study aimed to evaluate the neutrophilic enzymes in saliva **(Study III)** and GCF **(Study IV)** during pregnancy and post-partum. The periodontium, including gingival epithelial cells (Nebel *et al*., 2011), gingival fibroblasts (Kawahara and Shimazu, 2003), and periodontal ligament cells (Jönsson, 2007; Mamalis *et al*., 2011), as well as PMN and mononuclear lymphocytes (Szekeres-Bartho *et al*., 2001; Stygar *et al*., 2006) harbour estrogen and/or progesterone receptors. Various effects of estrogen on neutrophils, such as impaired functions (Björksten *et al*., 1978; Crouch *et al*., 1995), suppressed leukocyte production in the bone marrow (Josefsson *et al*., 1992), and diminished distribution of PMN cells in peripheral blood, occur during pregnancy. Therefore, defining the contribution of neutrophilic proteinases in the pathogenesis of pregnancy gingivitis provides awareness of underlying mechanisms in this special condition.

Table 7. Summary of the salivary data in the pregnant (Pr) study population and non-pregnant (N-Pr) control population during the follow-up. The results are given as medians (minimum-maximum). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

		Visits					
Marker	Study	PrEx I	PrEx II	PrEx III	PrEx IV	PrEx V	N-PrEx
Estradiol (pg/ml)		20.2 (5.0-51.1) ***	45.9 (12.7-128.3) *	49.4 (19.2-111.7) ***	2.2 (0.4-5.0)	2.7 (1.0-4.8)	2.5 (0.7-4.3)
Elastase (ng/ml)	III	330.3 (46.0-1627.0)	225.3 (29.2-1527.2)	178.3 (44.7-1168.9)	175.6 (88.1-1827.7)	176.7 (59.8-1357.1	251.9 (38.4-1425.8)
MMP-2 (IU)	III	0.7 (0.1-2.9)	0.7 (0.3-2.6)	0.6 (0.2-1.9)	0.8 (0.0-2.2)	0.8 (0.0-2.2)	0.8 (0.2-1.5)
MMP-8 (ng/ml)	III	240.2 (37.4-998.5) *	171.1 (25.4-899.5)	143.6 (28.8-907.0) *	368.9 (72.9-2304.2)	279.5 (54.4-944.1)	301.7 (15.9-1872.0)
MMP-9 (IU)	III	5.9 (1.2-13.6)	6.1 (2.1-10.6)	6.3 (3.5-11.0)	6.6 (2.1-10.5)	6.5 (4.1-16.6) **	9.1 (5.8-13.3)
MPO (ng/ml)	III	458.8 (74.9-1062.0)	303.1 (56.5-1065.2)	318.8 (99.7-986.3) ***	622.1 (148.9-1052.5)	367.1 (144.1-932.9)	319.7 (43.2-1051.3)
TIMP-1 (ng/ml)	III	81.3 (25.1-198.2)	82.5 (29.4-176.8)	84.0 (29.2-192.0)	71.2 (32.7-163.4)	99.9 (30.5-231.3)	112.3 (56.1-211.0)
Salivary flow rate (ml/min)		1.6 (0.8-3.6)	2.0 (1.0-3.4)	2.1 (1.0-3.4)	2.0 (1.0-3.0)	1.9 (0.8-3.4)	1.9 (1.0-3.8)
P. intermedia sensu lato (CFU/ml)	II	2.5×10^5 ($0-3.2 \times 10^6$)	1.7×10^5 ($0-2.2 \times 10^6$)	1.5×10^5 ($0-1.6 \times 10^6$)	3.7×10^5 ($0-6.0 \times 10^6$) *	1.5×10^5 ($0-5.0 \times 10^6$)	1.9×10^5 ($0.02-3.4 \times 10^6$)

5.4.1 Salivary proteinase levels (III)

The salivary findings are summarised in **Table 7.** Elastase levels decreased during pregnancy and post-partum with a similar trend as VPI scores; however, this decrease was not statistically significant. MMP-8 and MPO levels, in addition to the MMP-8/TIMP-1 ratio, increased significantly after delivery, whereas TIMP-1 levels remained significantly lower during pregnancy and after delivery in comparison with those observed in non-pregnant women. After lactation ended, all salivary enzymes, except MMP-9, returned to the same level as seen in the N-Pr group.

These results indicated that, despite increased BOP and PPD scores in the second trimester, a significant reduction of MMPs, MPO, and TIMP-1 expression occurs in periodontal tissues during pregnancy. Their reduced expression, in comparison with that of the non-pregnant controls, is further reflected in saliva. As the salivary flow rate remained stable during the follow-up, this cannot explain the reduction of proteinase and its inhibitor levels. More likely, this is, at least partly, due to the impaired neutrophil function during pregnancy (Björksten *et al*., 1978; Crouch *et al*., 1995). In fact, reduced microbial killing from the end of the first trimester (El-Maallem and Fletcher, 1980) and chemotaxis and phagocytosis by neutrophils from the beginning of the second trimester (Persellin and Thoi, 1979; Krause *et al*., 1987) seem to last till the end of pregnancy and then to return to the same level as seen in non-pregnant women within six weeks (El-Maallem and Fletcher, 1980; Crouch *et al*., 1995) up to three months after delivery (Krause *et al*., 1987). In **study III**, especially MMP-8 and MPO levels were reduced during pregnancy, and they reached the highest levels 4-6 weeks after delivery.

Since blood samples were not collected, it was not possible to confirm the suggested neutrophil impairment in the present study. Thus, the real origin of these enzymes present in saliva is still unclear, as other cell types besides neutrophils are able to produce the same proteinases. According to previous studies, however, PMN cells are the main source of deriving and releasing these enzymes in the oral cavity and saliva (Gangbar *et al*., 1990; Ingman *et al*., 1993; 1994; Uitto *et al*., 1990; 1996).

Furthermore, predominant MMPs in the pathogenesis of periodontitis are neutrophil-derived MMP-8 (collagenase-2) and MMP-9 (gelatinase B), fibroblastic MMP-2 (gelatinase A) and epithelial cell or bone-cell-derived MMP-13 (collagenase-3) (Borregaard *et al*., 1993). In addition to MMPs, neutrophils secrete two other antibacterial enzymes, PMN elastase (Belaaouaj, 2002) and MPO (Odeberg and Olsson, 1976). PMN elastase and MPO show interesting interrelationships with each other and with neutrophilic MMPs; elastase activates pro-MMP-2 (Shamamian *et al*., 2001) and potentiates the antimicrobial activity of MPO (Odeberg and Olsson, 1976), while MPO oxidatively activates MMP-8 (Sorsa *et al*., 2006). Among other MMPs, collagenases (MMP-1, MMP-8, and MMP-13) degrade type I collagen, the main collagen type in the periodontium, in fibrillar form, whereas MMP-2 and -9 contribute to the degradation of denatured collagen, and, in addition, MMP-2 digests fibrillar collagen.

During pregnancy, MMPs play an indisputable role in the regulation of the decidua, fetal membranes, and amniotic fluid (Weiss *et al.*, 2007). MMP-9 is responsible for the gelatinolytic activity in membranes during normal and pathological labour and separation of the placenta from the uterine wall at the end of labour, and thus is barely detectable until delivery (Demir-Weusten *et al*., 2007), while MMP-8 is involved in the cervical ripening process. The highest levels are found in cervical biopsies post-partum (Sennström *et al*., 2003). Its increase in amniotic fluid reflects the presence of intra-amniotic infection, which may result in preterm contractions and preterm birth (Angus *et al*., 2001). Therefore, the suppression of MMPs in periodontal tissues during pregnancy can be seen as a protective mechanism, which may inhibit preterm deliveries.

5.4.2 Neutrophilic enzyme levels in GCF (IV)

In **study IV**, all clinical measurements and GCF samples were collected from the same sites, the mesio-buccal site of the first (or if missing, second) molars. These site-specific clinical data responded similarly to the subject-based clinical observations in **study I**. As a brief summary, the number and proportions of sites with BOP and PPD increased significantly ($p<0.001$) during the second trimester, while VPI scores decreased. From mid-pregnancy onwards, BOP and PPD scores decreased significantly visit by visit, reaching the same level as observed in the N-Pr group after lactation. PMN elastase levels followed the same trend as VPI scores, *i.e*. they decreased throughout pregnancy. MPO and MMP-8 levels were steady during pregnancy, regardless of the increase in BOP scores in the second trimester. Both neutrophilic enzymes increased after delivery, but decreased until the end of lactation. The GCF-derived findings are summarised in **Table 8.**

Neutrophils play a major role in the innate defence against bacterial challenge, and neutrophil-derived antibacterial peptide and enzyme levels increase with the initiation of infection and inflammation (Uitto *et al*., 2003). Therefore, several markers of neutrophilic activity, including neutrohilic elastase, MPO, and MMP-8, have been used as reliable markers of periodontal disease (Söder *et al*., 2002; Loos and Tjoa, 2005). The levels of these enzymes and their inhibitors in GCF reflect the periodontal disease status (Eley and Cox, 1996; Tervahartiala *et al*., 2000; Sorsa *et al*., 2004; 2006). In fact, PMN elastase is responsible for degraded host extracellular matrix components and initial tissue breakdown not only at the beginning of the periodontal destruction *(Ujiie et al.*, 2007), but also throughout the disease progression PMN elastase levels in GCF rise (Armitage *et al*., 1994; Eley and Cox, 1996). Neutrophilic elastase and MPO are broad-range antibacterial agents and, in addition, elastase can degrade the extracellular matrix and connective tissue components together with MMP-8, while MMP-8 is activated by MPO (Uitto *et al*., 2003). Neutrophilic elastase and several MMPs play an important role in delivery by regulating the membrane rupture, and their excessive increase during pregnancy may initiate a preterm birth (Weiss *et al.,* 2007). To minimize the risk of preterm births, the immune response is suppressed until delivery (Weinberg, 1987). According to this, it can be suggested, that the low MPO, MMP-8, and elastase levels during pregnancy **in study IV** were not only related to local conditions, but were also the outcome of a suppressed immune condition. This is likely, since their levels increased after delivery, as has been shown previously (El-

Maallem and Fletcher, 1980; Cunze *et al*., 1998; Weiss *et al.,* 2007). However, it should be kept in mind that the suppression of these neutrophilic markers does not necessarily occur at a transcriptional level; MPO and elastase, which are localised in granules of neutrophils in the non-pregnant state, accumulate at the neutrohilic membrane during pregnancy (Kindzelskii *et al*., 2006). This exclusive and yet unexplained phenomenon leads to diminished neutrophilic phagocytosis. Therefore, it is important to understand that pregnancy is a unique condition and the immune response acts very differently from that in the non-pregnant condition.

As has been shown in recent studies, pregnancy gingivitis does not increase the cytokine and prostaglandin secretion (Bieri *et al*., 2012 Carrillo-de-Albornoz *et al*., 2012), which activates the neutrophils. This might be another explanation of why the reduced neutrophil activity does not result in periodontal soft tissue or bone destruction. During the follow-up, no changes in CAL or bone damage were observed. Bostanci *et al*. (2007) found alterations in the RANKL/OPG ratio in GCF between healthy subjects and patients with gingivitis or periodontitis, and a positive correlation between the RANKL/OPG ratio to PPD and CAL. However, estrogen can modulate the tissue maintenance and repair by increasing the OPG expression in periodontal ligament cells (Liang *et al*., 2008). Furthermore, estrogen negatively regulates the secretions of several cytokines, such as IL-1, -6, and TNF-α, which are related to prolonged survival of the osteoclasts. This may explain why bone is supported during pregnancy.

Table 8. Summary of the subgingival and GCF-related data in pregnant (Pr) study population and non-pregnant (N-Pr) control population during the follow-up. The results are given as medians (minimum-maximum). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

		Visits					
Marker	Study	**PrEx I**	**PrEx II**	**PrEx III**	**PrEx IV**	**PrEx V**	**N-PrEx**
Elastase/site (ng/30 s)	IV	2.3 (0.0-162.3)	3.3 (0.0-167.0) *	1.7 (0.0-194.6) ***	0.9 (0.0-12.5) **	1.4 (0.1-99.8) **	2.1 (0.5-41.7)
MMP-8/site (ng/30 s)	IV	14.6 (0.1-271.1)	13.6 (0.0-262.7)	14.7 (0.0-170.5)	20.7 (0.2-94.4) **	15.6 (1.3-73.5)	17.8 (3.4-63.1)
MPO/site (ng/30 s)	IV	31.2 (0.0-1039.2)	24.5 (0.0-79.1)	30.4 (0.0-1117.7) *	38.5 (0.3-725.9) *	20.8 (0.0-650.4)	26.0 (3.2-169.8)
TIMP-1/site (ng/30 s)	IV	0.0 (0.0-1.8)	0.0 (0.0-3.5)	0.0 (0.0-2.5)	0.0 (0.0-1.8)	0.0 (0.0-2.1) *	0.0 (0.0-1.0)
P. intermedia sensu lato (CFU/ml)	II	5.0×10^4 ($0-7.2 \times 10^5$)	3.7×10^4 ($0-2.6 \times 10^6$)	1.2×10^4 ($0-1.1 \times 10^6$) *	6.1×10^4 ($0-2.1 \times 10^6$)	3.1×10^4 ($0-6.5 \times 10^5$)	3.8×10^4 ($0-3.2 \times 10^{5)}$)

6. SUMMARY AND CONCLUSIONS

Pregnancy gingivitis is an exacerbated tissue response against a relatively limited amount of plaque. Elevated concentrations of female sex hormones during pregnancy modify the quality of the microbial dental biofilm, but also mediate the level of the host response by regulating the participation of immune-inflammatory cells and their destructive enzymes to the gingival disease pathogenesis. The current study, using a prospective longitudinal follow-up design, evaluated the clinical, microbial, and enzymatic changes at periodontal sites and in saliva during pregnancy and post-partum, aiming to elucidate interactions between the pregnancy status, microbial challenge, and host response.

The main findings are summarised below and in **Figure 12**:

Pregnancy-related changes in clinical parameters (I)

- Despite decreasing plaque scores after the first trimester, BOP% and PD increased significantly during the 2nd trimester
- No changes in CAL during the follow-up
- After lactation, women in the Pr group had similar VPI%, BOP%, and PD scores as women in the N-Pr group

Microbial changes during pregnancy (II)

- Increased levels of *P. nigrescens* were associated with pregnancy gingivitis
- *P. intermedia* sensu stricto was rarely found in these women without periodontitis

Host response during pregnancy (III, IV, unpublished data)

- No changes in salivary flow rates during pregnancy
- Host enzymes and their inhibitors were secreted into saliva and GCF at low levels during pregnancy
- After lactation, enzyme levels returned to the same level as seen in non-pregnant women

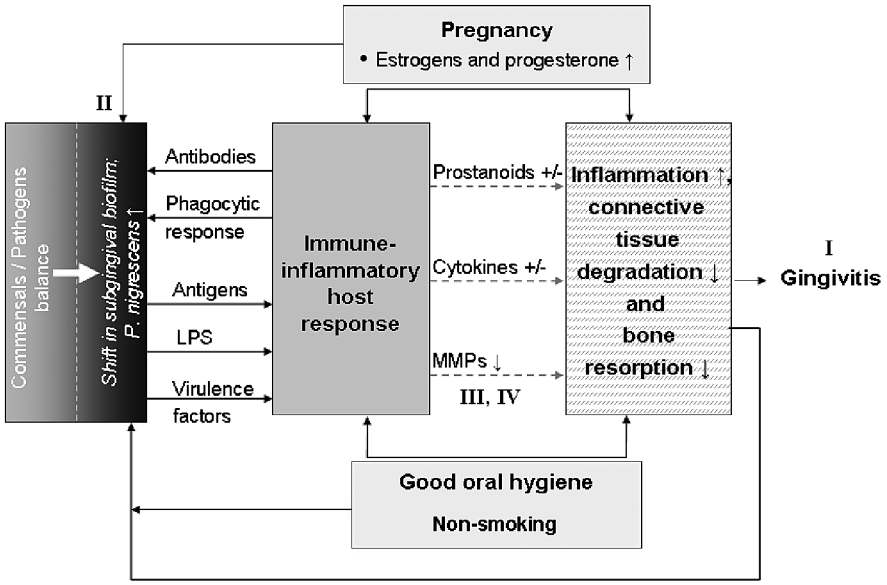


Figure 12. Summary of the key findings from studies I-IV. (Modified from Page and Kornman, 1997).

6.1 CONCLUSIONS

It is notable that the level of periodontal inflammation and presence of pathogenic biofilm before the initiation of pregnancy, smoking, older age, and diabetes may influence the severity of gingival tissue response during pregnancy. Therefore, the conclusions are based on the findings in generally healthy, non-smoking women without previous diagnosis of periodontitis. The current study confirms that, in the presence of adequate oral hygiene, a low level of bacteria can initiate gingivitis within elevated female sex hormone concentrations during pregnancy. Furthermore, the enhanced susceptibility to aggravated gingival inflammation especially during the second trimester is due to the increased levels of *P. nigrescens* in subgingival plaque and decreased levels of neutrophilic enzymes in GCF and in saliva. Nevertheless, after lactation, clinical, microbiological and host response states return to those seen in non-pregnant controls. Thus, gingival inflammation is reversible and does not proceed to periodontal breakdown.

6.2 FUTURE ASPECTS

This study series revealed new aspects of the contribution of *P. intermedia*-group organisms and suppressed host response in pregnancy-related changes in the periodontium. In relatively young, generally healthy, and non-smoking women without a previous diagnosis of periodontitis, the pregnancy itself does not predispose to periodontitis. This leads to speculations about other defence systems which replace the suppressed neutrophilic response. Additional mechanisms may be involved in the protection of bone from excessive degradation during pregnancy. Therefore,

further studies are needed to define factors related to the innate defence mechanism, including epithelial defensins and bone degradation inhibition, e.g. inhibition of RANKL formation by sex steroid hormones. Furthermore, clinical studies with multi-centre study designs and site-specific data analysis will add power to these analyses.

ACKNOWLEDGEMENTS

This study has been carried out at the Kerava Municipal Health Care Centre, at the Anaerobe Reference Laboratory, the National Public Health Institute (KTL) / currently the National Institute for Health and Welfare (THL), at the Biomedicum Research Laboratory, Institute of Dentistry, University of Helsinki, and at the Department of Periodontology, Institute of Dentistry, University of Turku, Finland. All the former and current Chief Dental Officers, Heads, and Deans of these institutes are warmly acknowledged for providing excellent working facilities during the last decade. In addition, the PeGaSOS postgraduate school is warmly acknowledged for enabling my participation in the postgraduate program.

I am grateful to all of you, who have given me any advice and support during this academic journey. Particularly, I would like to express my deepest gratitude to the following persons:

My official supervisors, Professor Eija Könönen and Professor Timo Sorsa, and co-supervisor Riitta Pajukanta, DDS, PhD, for their excellent guidance, motivation, and constant belief in me throughout these years. I am extremely thankful to Riitta and Timo for their offer to initiate this PhD study. Riitta has shared her clinical knowledge, while Timo has supported this long journey not only with his knowledge and experience in periodontal biochemistry, but also with the never-ending optimism. Most of all, I have had the extreme privilege of Eija´s unlimited support in every part of this thesis project. During these years, she has unselfishly shared her microbial and clinical expertise, used countless number of hours editing my numerous abstracts, grant applications, and manuscripts, and guided my steps in the academic staircase, for which I am forever grateful.

All my co-authors of the original publications, Gunnstein Haraldsson, PhD, Mari Hyvönen, and Docent Taina Tervahartiala, for their efforts and contributions.

Professor Tellervo Tervonen, and Docent Oskari Heikinheimo for their critical review of this thesis. Their valuable comments and suggestions, which improved the thesis at its final stage, are highly appreciated.

The members of my follow-up committee, Professor Yrsa Le Bell and Professor Timo Närhi, for their kind encouragement and support.

All my former and current colleagues, dental nurses, and dental hygienists at the Dental Clinic, Kerava Municipal Health Care Centre for maintaining a positive and cheerful working atmosphere. I owe my special thanks to my colleagues Angelina Allen and Marika Heikkilä for their support and friendship, but also for their kind help in the recruitment of non-pregnant women, and to midwife Marjo Niinisalo among the other nurses at the Prenatal Clinic, Kerava Municipal Health Care Centre for their assistance in the recruitment of pregnant women.

All pregnant and non-pregnant women, who took part in this follow-up study, for their time and interest. This study could not succeed without your commitment!

The "ANBA-family" at the KTL Anaerobe Reference laboratory. The late Professor Hannele "Haba" Jousimies-Somer, Silja Mentula, PhD, Anne Bryk, and Arja Kanervo-Nordström, are acknowledged with great respect for their advice and guidance, and Marja Piekkola, Anne Rinta-Opas, and Ani Sarpi for technical assistance during my "ANBA years".

The working mates and research colleagues at the Biomedicum research laboratory, University of Helsinki. Especially, I wish to thank, Kirsti Kari, MSc, the Head of the Scientific Laboratory, for her guidance, Ritva Keva for technical assistance, and Docent Päivi Mäntylä for sharing her experience.

The personnel at the Institute of Dentistry, University of Turku for a very warm and supporting atmosphere. In particular, I would like to thank Anna Haukioja, PhD, Docent Merja Laine, Professor Stina Syrjänen, Assistant Professor Anna-Liisa Svedström-Oristo, Docent Eva Söderling, Docent Arzu Tezvergil-Mutluay, Assistant Professor Helena Yli-Urpo, for sharing their interest and giving practical advice during these last months before the dissertation day, and Karin Kanerva, Maarit Tuhkanen, and Annukka Wallenius for practical assistance in the final arrangements.

Jacqueline Välimäki, MA, for revising the English language of the thesis, and Tero Vahlberg, MSc, for providing support in statistics. Senior Administrative Officer Outi Irjala and Clerical Officer Riitta Paju for their kind help and technical advice related with the PhD studies.

Personnel of the Clinic of Oral Diseases, Turku University Hospital, and Personnel of the Turku Health Care Centre for all their interest and empathy. Especially my former and current specialists trainee colleagues Hannaleena Havia, Anne Keinänen, Nina Mandelin, Kirsi Mikkola, Marja Pylkki, Elina Rissa, and Leeni Sadam are acknowledged for sharing the study load and the pleasant discussions during the last two years.

Families Könönen and Uitto for their friendship and support. Especially, Inne Uitto and Laura Könönen are acknowledged for giving so much happiness to our lives with art, when our minds have been full of science.

Last but definitely not least, my family for their most valuable support, never-ending encouragement and love. Words are not enough to describe how much these have meant to me during these years. I love you all! My heartfelt thanks are dedicated to my dear husband Ulvi and our sunshines, "Eppuli" and "Emmentaali", for making my life so rich and joyful!

The financial support from the Academy of Finland, the Finnish Dental Society Apollonia, the Finnish Association of Women Dentists, the HUCH-EVO-Foundation, the Paulo Foundation, the Postgraduate School of Oral Health Science (PeGaSOS), and Turku Dental Society is also greatly appreciated and gratefully acknowledged.

Turku, November 2012

Merri Gürsoy

REFERENCES

Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE (2005). Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol* 43:5721-5732.

Adriaens LM, Alessandri R, Spörri S, Lang NP, Persson GR (2009). Does pregnancy have an impact on the subgingival microbiota? *J Periodontol* 80:72-81.

Aimes RT, Quigley JP (1995). Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. *J Biol Chem* 270:5872-5876.

Amar S, Chung KM (1994). Influence of hormonal variation on the periodontium in women. *Periodontol 2000* 6:79-87.

Angus SR, Segel SY, Hsu CD, Locksmith GJ, Clark P, Sammel MD, *et al.* (2001). Amniotic fluid matrix metalloproteinase-8 indicates intra-amniotic infection. *Am J Obstet Gynecol* 185:1232-1238.

Arafat AH (1974). Periodontal status during pregnancy. *J Periodontol* 45:641–643.

Armitage GC, Jeffcoat MK, Chadwick DE, Taggart EJ, Jr., Numabe Y, Landis JR, *et al*. (1994). Longitudinal evaluation of elastase as a marker for the progression of periodontitis. *J Periodontol* 65:120-128.

Armitage GC (1999). Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 4:1-6.

Armitage GC (2002). Classifying periodontal diseases--a long-standing dilemma. *Periodontol 2000* 30:9-23.

Barros SP, Wirojchanasak S, Barrow DA, Panagakos FS, Devizio W, Offenbacher S (2010). Triclosan inhibition of acute and chronic inflammatory gene pathways. *J Clin Periodontol* 37:412-418.

Belaaouaj A (2002). Neutrophil elastase-mediated killing of bacteria: lessons from targeted mutagenesis. *Microbes Infect* 4:1259-1264.

Bieri RA, Adriaens L, Spörri S, Lang NP, Persson GR (2012). Gingival fluid cytokine expression and subgingival bacterial counts during pregnancy and postpartum: a case series. *Clin Oral Investig* Jan 17. [Epub ahead of print]

Borregaard N, Lollike K, Kjeldsen L, Sengeløv H, Bastholm L, Nielsen MH, et al. (1993). Human neutrophil granules and secretory vesicles. *Eur J Haematol* 51:187-198.

Björksten B, Söderström T, Damber MG, von Schoultz B, Stigbrand T (1978). Polymorphonuclear leucocyte function during pregnancy. *Scand J Immunol* 8:257-262.

Bosshardt DD, Lang NP (2005). The junctional epithelium: from health to disease. *J Dent Res* 84:9-20.

Bostanci N, Ilgenli T, Emingil G, Afacan B, Han B, Töz H, *et al*. (2007). Gingival crevicular fluid levels of RANKL and OPG in periodontal diseases: implications of their relative ratio. *J Clin Periodontol* 34:370-376.

Bouman A, Heineman MJ, Faas MM (2005). Sex hormones and the immune response in humans. *Hum Reprod Update* 11:411-423.

Brabin BJ (1985). Epidemiology of infection in pregnancy. *Rev Infect Dis* 7:579-603.

Buduneli N, Baylas H, Buduneli E, Türkoğlu O, Dahlen G (2005). Evaluation of the relationship between smoking during pregnancy and subgingival microbiota. *J Clin Periodontol* 32:68-74.

Buduneli N, Becerik S, Buduneli E, Baylas H, Kinnby B (2010). Gingival status, crevicular fluid tissue-type plasminogen activator, plasminogen activator inhibitor-2 levels in pregnancy versus post-partum. *Aust Dent J* 55:292-297.

Carrillo-de-Albornoz A, Figuero E, Herrera D, Bascones-Martínez A (2010). Gingival changes during pregnancy: II. Influence of hormonal variations on the subgingival biofilm. *J Clin Periodontol* 37:230-240.

Carrillo-de-Albornoz A, Figuero E, Herrera D, Cuesta P, Bascones-Martínez A (2012). Gingival changes during pregnancy: III. Impact of clinical, microbiological, immunological and socio-demographic factors on gingival inflammation. *J Clin Periodontol* 39:272-283.

Chaikin BS (1977). Incidence of gingivitis in pregnancy. *Quintessence Int Dent Dig* 8:81–89.

Chan YH (2003a). Biostatistics 101: data presentation. *Singapore Med J* 44:280-285.

Chan YH (2003b). Biostatistics 102: quantitative data--parametric & non-parametric tests. *Singapore Med J* 44:391-396.

Chan YH (2003c). Biostatistics 103: qualitative data - tests of independence. *Singapore Med J* 44:498-503.

Chan YH (2003d). Biostatistics 104: correlational analysis. *Singapore Med J* 44:614-619.

Chaves ES, Wood RC, Jones AA, Newbold DA, Manwell MA, Kornman KS (1993). Relationship of "bleeding on probing" and "gingival index bleeding" as clinical parameters of gingival inflammation. *J Clin Periodontol* 20:139-143.

Chen SJ, Liu YL, Sytwu HK (2012). Immunologic regulation in pregnancy: from mechanism to therapeutic strategy for immunomodulation. *Clin Dev Immunol* 2012:258391.

Cohen DW, Friedman L, Shapiro J, Kyle GC (1969). A longitudinal investigation of the periodontal changes during pregnancy. *J Periodontol* 40:563-570.

Cohen DW, Shapiro J, Friedman L, Kyle GC, Franklin S (1971). A longitudinal investigation of the periodontal changes during pregnancy and fifteen months post-partum. II. *J Periodontol* 42:653-657.

Crouch SP, Crocker IP, Fletcher J (1995). The effect of pregnancy on polymorphonuclear leukocyte function. *J Immunol* 155:5436-5443.

Cunze T, Osmers R, Herzog S, Speer C, Kuhn W (1998). Changes in plasma elastase during pregnancy and sub partu. *Gynecol Obstet Invest* 45:89-92.

Dahlén G, Pipattanagovit P, Rosling B, Möller ÅJ (1993). A comparison of two transport media for saliva and subgingival samples. *Oral Microbiol Immunol* 8:375-382.

Darne J, McGarrigle HH, Lachelin GC (1987). Saliva oestriol, oestradiol, oestrone and progesterone levels in pregnancy: spontaneous labour at term is preceded by a rise in the saliva oestriol:progesterone ratio. *Br J Obstet Gynaecol* 94:227-235.

Davies RM, Ellwood RP, Davies GM (2004). The effectiveness of a toothpaste containing triclosan and polyvinyl-methyl ether maleic acid copolymer in improving plaque control and gingival health: a systematic review. *J Clin Periodontol* 31:1029-1033.

Demir-Weusten AY, Seval Y, Kaufmann P, Demir R, Yucel G, Huppertz B (2007). Matrix metalloproteinases-2, -3 and -9 in human term placenta. *Acta Histochem* 109:403-412.

Demmer RT, Papapanou PN, Jacobs DR Jr, Desvarieux M (2008). Bleeding on probing differentially relates to bacterial profiles: the Oral Infections and Vascular Disease Epidemiology Study. *J Clin Periodontol* 35:479-486.

Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner ACR, Yu WH, *et al*. (2010). The human oral microbiome. *J Bacteriol* 192:5002-5017.

Eley BM, Cox SW (1996). A 2-year longitudinal study of elastase in human gingival crevicular fluid and periodontal attachment loss. *J Clin Periodontol* 23:681-692.

El-Maallem H, Fletcher J (1980). Impaired neutrophilic function and myeloperoxidase deficiency during pregnancy. *Br J Haematol* 44:375-381.

Emingil G, Afacan B, Tervahartiala T, Töz H, Atilla G, Sorsa T (2008). Gingival crevicular fluid and serum matrix metalloproteinase-8 and tissue inhibitor of matrix metalloproteinase-1 levels in renal transplant patients undergoing different immunosuppressive therapy. *J Clin Periodontol* 35:221-229.

Ereş G, Altıok E, Ozkul A, Açıkel CH (2011). Subgingival Epstein-Barr and cytomegalovirus occurrence in pregnancy gingivitis. *J Periodontol* 82:1676-1684.

Faurschou M, Borregaard N (2003). Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* 5:1317-1327.

Figuero E, Carrillo-de-Albornoz A, Herrera D, Bascones-Martinez A (2010). Gingival changes during pregnancy: I. Influence of hormonal variations on clinical and immunological parameters. *J Clin Periodontol* 37:220-229.

Gaffar A, Scherl D, Afflitto J, Coleman EJ (1995). The effect of triclosan on mediators of gingival inflammation. *J Clin Periodontol* 22:480-484.

Galgut P (1999). A comparison of different indices used in the clinical assessment of plaque and gingival bleeding. *Clin Oral Invest* 3:96-99.

Gangbar S, Overall CM, McCulloch CA, Sodek J (1990). Identification of polymorphonuclear leukocyte collagenase and gelatinase activities in mouthrinse samples: correlation with periodontal disease activity in adult and juvenile periodontitis. *J Periodontal Res* 25:257-267.

Garlet GP, Cardoso CR, Silva TA, Ferreira BR, Avila-Campos MJ, Cunha FQ, *et al*. (2006). Cytokine pattern determines the progression of experimental

periodontal disease induced by *Actinobacillus actinomycetemcomitans* through the modulation of MMPs, RANKL, and their physiological inhibitors. *Oral Microbiol Immunol* 21:12-20.

Goodson JM, Tanner AC, Haffajee AD, Sornberger GC, Socransky SS (1982). Patterns of progression and regression of advanced destructive periodontal disease. *J Clin Periodontol* 9:472-481.

Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P, *et al*. (1986). Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* 320:134-139.

Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J (1986). Sequence and expression of human estrogen receptor complementary DNA. *Science* 231:1150-1154.

Guthmiller JM, Hassebroek-Johnson JR, Weenig DR, Johnson GK, Kirchner HL, Kohout FJ, *et al.* (2001). Periodontal disease in pregnancy complicated by type 1 diabetes mellitus. *J Periodontol* 72:1485-1490.

Guyton AC (1991). Pregnancy and lactation. In *Textbook of medical physiology*. 8th edn. Philadelphia, W.B. Saunders Company: 915-928.

Gürsoy M, Gürsoy UK, Sorsa T, Pajukanta R, and Könönen E (2011). Plaque accumulation and estrogen levels predict gingival inflammation during pregnancy. *J Dent Res* 90 (Spec Iss B): 289.

Haffajee AD, Socransky SS (2001). Relationship of cigarette smoking to the subgingival microbiota. *J Clin Periodontol* 28:377-388.

Hanemaaijer R, Sorsa T, Konttinen YT, Ding Y, Sutinen M, Visser H, *et al*. (1997). Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblasts and endothelial cells. Regulation by tumor necrosis factor-alpha and doxycycline. *J Biol Chem* 272:31504-31509.

Hansen PJ (1998). Regulation of uterine immune function by progesterone--lessons from the sheep. *J Reprod Immunol* 40:63-79.

Haraldsson G, Holbrook W (1999). Identifying clinically important gram-negative anaerobes from the oral cavity. *Eur J Oral Sci* 107:429-436.

Hassel TM (1993). Tissues and cells of the periodontium. *Periodontol 2000* 3:9-38.

Hasson E (1960). Pregnancy gingivitis. *Harefuah* 58:224–226.

Heikkinen AM, Sorsa T, Pitkäniemi J, Tervahartiala T, Kari K, Broms U, *et al.* (2010). Smoking affects diagnostic salivary periodontal disease biomarker levels in adolescents. *J Periodontol* 81:1299-1307.

Hemmilä I, Dakubu S, Mukkala VM, Siitari H, Lövgren T (1984). Europium as a label in time-resolved immunofluorometric assays. *Anal Biochem* 137:335-343.

Hernández Ríos M, Sorsa T, Obregón F, Tervahartiala T, Valenzuela MA, Pozo P, *et al*. (2009). Proteolytic roles of matrix metalloproteinase (MMP)-13 during progression of chronic periodontitis: initial evidence for MMP-13/MMP-9 activation cascade. *J Clin Periodontol* 36: 1011-1017.

Hioe KP, van der Weijden GA (2005). The effectiveness of self-performed mechanical plaque control with triclosan containing dentifrices. *Int J Dent Hyg* 3:192-204.

Hugoson A (1971). Gingivitis in pregnant women. A longitudinal clinical study. *Odontol Revy* 22:65-84.

Ingman T, Sorsa T, Konttinen YT, Liede K, Saari H, Lindy O, *et al*. (1993). Salivary collagenase, elastase- and trypsin-like proteases as biochemical markers of periodontal tissue destruction in adult and localized juvenile periodontitis. *Oral Microbiol Immunol* 8:298-305.

Ingman T, Sorsa T, Lindy O, Koski H, Konttinen YT (1994). Multiple forms of gelatinases/type IV collagenases in saliva and gingival crevicular fluid of periodontitis patients. *J Clin Periodontol* 21:26-31.

Ingman T, Tervahartiala T, Ding Y, Tschesche H, Haerian A, Kinane DF, *et al.* (1996). Matrix metalloproteinases and their inhibitors in gingival crevicular fluid and saliva of periodontitis patients. *J Clin Periodontol* 23:1127-1132.

Ito I, Hayashi T, Yamada K, Kuzuya M, Naito M, Iguchi A (1995). Physiological concentration of estradiol inhibits polymorphonuclear leukocyte chemotaxis via a receptor mediated system. *Life Sci* 56:2247-2253.

Jafarzadeh H, Sanatkhani M, Mohtasham N (2006). Oral pyogenic granuloma: a review. *J Oral Sci* 48:167-175.

Jakubovics NS, Kolenbrander PE (2010). The road to ruin: the formation of disease-associated oral biofilms. *Oral Dis* 16:729-739.

Jensen J, Liljemark W, Bloomquist C (1981). The effect of female sex hormones on subgingival

plaque. *J Periodontol* 52:599-602.

Jonsson R, Howland BE, Bowden GH (1988). Relationships between periodontal health, salivary steroids, and *Bacteroides intermedius* in males, pregnant and non-pregnant women. *J Dent Res* 67:1062-1069.

Josefsson E, Tarkowski A, Carlsten H (1992). Anti-inflammatory properties of estrogen. I. In vivo suppression of leukocyte production in bone marrow and redistribution of peripheral blood neutrophils. *Cell Immunol* 142:67-78.

Joss A, Adler R, Lang NP (1994). Bleeding on probing. A parameter for monitoring periodontal conditions in clinical practice. *J Clin Periodontol* 21:402–408.

Jousimies-Somer H, Summanen P, Citron DM, Baron EJ, Wexler HM, Finegold SM (2002). *Wadsworth–KTL Anaerobic Bacteriology Manual*, 6th edn. Belmont, CA, Star Publishing.

Jönsson D (2007). The biological role of the female sex hormone estrogen in the periodontium--studies on human periodontal ligament cells. *Swed Dent J Suppl* 187:11-54.

Kawahara K, Shimazu A (2003). Expression and intracellular localization of progesterone receptors in cultured human gingival fibroblasts. *J Periodontal Res* 38:242-246.

Kindzelskii AL, Clark AJ, Espinoza J, Maeda N, Aratani Y, Romero R, *et al.* (2006). Myeloperoxidase accumulates at the neutrophil surface and enhances cell metabolism and oxidant release during pregnancy. *Eur J Immunol* 36:1619-1628.

Klebanoff SJ (2005). Myeloperoxidase: friend and foe. *J Leukoc Biol* 77:598-625.

Knuuttila M, Suominen-Taipale L (2008). Periodontal status. In Suominen-Taipale L, Nordblad A, Vehkalahti M, Aromaa A (eds). Oral health in the Finnish adult population, Health 2000 Survey. Helsinki. *Publications of the National Public Health Institute* B25/2008:49-53.

Kornman KS, Loesche WJ (1980). The subgingival microbial flora during pregnancy. *J Periodontal Res*15:111-122.

Kornman KS, Loesche WJ (1982). Effects of estradiol and progesterone on *Bacteroides melaninogenicus* and *Bacteroides gingivalis*. *Infect Immun* 35:256-263.

Kraivaphan P, Amornchat C, Triratana T, Leethochawalit U (2006). Clinical effect of a triclosan containing dentifrice on gingivitis during pregnancy and post-partum. *Southeast Asian J Trop Med Public Health* 37:820-825.

Krause PJ, Ingardia CJ, Pontius LT, Malech HL, LoBello TM, Maderazo EG (1987). Host defense during pregnancy: neutrophil chemotaxis and adherence. *Am J Obstet Gynecol* 157:274-280.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A* 93:5925-5930.

Kumar PS, Leys EJ, Bryk JM, Martinez FJ, Moeschberger ML, Griffen AL (2006). Changes in periodontal health status are associated with bacterial community shifts as assessed by quantitative 16S cloning and sequencing. *J Clin Microbiol* 44:3665-3673.

Kunnen A, van Doormaal JJ, Abbas F, Aarnoudse JG, van Pampus MG, Faas MM (2010). Periodontal disease and pre-eclampsia: a systematic review. *J Clin Periodontol* 37:1075-1087.

Könönen E, Jousimies-Somer H, Asikainen S (1992). Relationship between oral gram-negative anaerobic bacteria in saliva of the mother and the colonization of her edentulous infant. *Oral Microbiol Immunol* 7:273-276.

Könönen E (1993). Pigmented *Prevotella* species in the periodontally healthy oral cavity. *FEMS Immunol Med Microbiol* 6:201-205.

Könönen E, Saarela M, Karjalainen J, Jousimies-Somer H, Alaluusua S, Asikainen S (1994). Transmission of oral *Prevotella melaninogenica* between a mother and her young child. *Oral Microbiol Immunol* 9:310-314.

Könönen E, Eerola E, Frandsen EV, Jalava J, Mättö J, Salmenlinna S, *et al.* (1998). Phylogenetic characterization and proposal of a new pigmented species to the genus *Prevotella*: *Prevotella pallens* sp. nov. *Int J Syst Bacteriol* 48:47-51.

Könönen E, Wolf J, Mättö J, Frandsen EV, Poulsen K, Jousimies-Somer H, *et al.* (2000). The *Prevotella intermedia* group organisms in young children and their mothers as related to maternal periodontal status. *J Periodontal Res* 35:329-334.

Könönen E, Paju S, Pussinen PJ, Hyvönen M, Di Tella P, Suominen-Taipale L, *et al.* (2007). Population-based study of salivary carriage of periodontal pathogens in adults. *J Clin Microbiol* 45:2446-2451.

Laine M, Tenovuo J, Lehtonen OP, Ojanotko-Harri

A, Vilja P, Tuohimaa P (1988). Pregnancy-related changes in human whole saliva. *Arch Oral Biol* 33:913-917.

Lamster IB, Oshrain RL, Fiorello LA, Celenti RS, Gordon JM (1988). A comparison of 4 methods of data presentation for lysosomal enzyme activity in gingival crevicular fluid. *J Clin Periodontol* 15:347-352.

Lamster IB (1997). Evaluation of components of gingival crevicular fluid as diagnostic tests. *Ann Periodontol* 2:123-137.

Lamster IB, Ahlo JK (2007). Analysis of gingival crevicular fluid as applied to the diagnosis of oral and systemic diseases. *Ann N Y Acad Sci* 1098:216-229.

Lang NP, Joss A, Orsanic T, Gusberti FA, Siegrist BE (1986). Bleeding on probing – a predictor for the progression of periodontal disease? *J Clin Periodontol* 13:590–596.

Lang NP, Adler R, Joss A, Nyman S (1990). Absence of bleeding on probing – an indicator of periodontal stability. *J Clin Periodontol* 17:714–721.

Lapp CA, Thomas ME, Lewis JB (1995). Modulation by progesterone of interleukin-6 production by gingival fibroblasts. *J Periodontol* 66:279-284.

Lapp CA, Lohse JE, Lewis JB, Dickinson DP, Billman M, Hanes PJ, *et al*. (2003). The effects of progesterone on matrix metalloproteinases in cultured human gingival fibroblasts. *J Periodontol* 74:277-288.

Liang L, Yu JF, Wang Y, Ding Y (2008). Estrogen regulates expression of osteoprotegerin and RANKL in human periodontal ligament cells through estrogen receptor beta. *J Periodontol* 79:1745-1751.

Lieff S, Boggess KA, Murtha AP, Jared H, Madianos PN, Moss K, et al. (2004). The oral conditions and pregnancy study: periodontal status of a cohort of pregnant women. *J Periodontol* 75:116-126.

Lindhe J, Brånemark PI (1967). Changes in vascular permeability after local application of sex hormones. *J Periodontal Res* 2:259-265.

Lindhe J, Brånemark PI, Lundskog J (1967). Changes in vascular proliferation after local application of sex hormones. *J Periodontal Res* 2:266-272.

Loos BG, Tjoa S (2005). Host-derived diagnostic markers for periodontitis: do they exist in gingival crevice fluid? *Periodontol 2000* 39:53-72.

Luppi P, Haluszczak C, Betters D, Richard CA, Trucco M, DeLoia JA (2002). Monocytes are progressively activated in the circulation of pregnant women. *J Leukoc Biol* 72:874-884.

Luppi P (2003). How immune mechanisms are affected by pregnancy. *Vaccine* 21:3352-3357.

Löe H, Silness J (1963). Periodontal disease in pregnancy. I. Prevalence and severity. *Acta Odontol Scand* 21:533–551.

Machuca G, Khoshfeiz O, Lacalle JR, Machuca C, Bullón P (1999). The influence of general health and socio-cultural variables on the periodontal condition of pregnant women. *J Periodontol* 70:779-785.

Malisa JE, Mosha HJ, Masalu JRP (1993). Periodontal status of pregnant and postpartum mothers aged 18–45 years attending MCH clinics in Tanga Municipality, Tanzania. *East Afr Med J* 70:799–802.

Mamalis A, Markopoulou C, Lagou A, Vrotsos I (2011). Oestrogen regulates proliferation, osteoblastic differentiation, collagen synthesis and periostin gene expression in human periodontal ligament cells through oestrogen receptor beta. *Arch Oral Biol* 56:446-455.

Mariotti A (1994). Sex steroid hormones and cell dynamics in the periodontium. *Crit Rev Oral Biol Med* 5:27-53.

Mascarenhas P, Gapski R, Al-Shammari K, Wang HL (2003). Influence of sex hormones on the periodontium. *J Clin Periodontol* 30:671-681.

Mealey BL, Moritz AJ (2003). Hormonal influences: effects of diabetes mellitus and endogenous female sex steroid hormones on the periodontium. *Periodontol 2000* 32:59-81.

Miyagi M, Aoyama H, Morishita M, Iwamoto Y (1992). Effects of sex hormones on chemotaxis of human peripheral polymorphonuclear leukocytes and monocytes. *J Periodontol* 63:28-32.

Miyagi M, Morishita M, Iwamoto Y (1993). Effects of sex hormones on production of prostaglandin E2 by human peripheral monocytes. *J Periodontol* 64:1075-1078.

Miyazaki H, Yamashita Y, Shirahama R, Goto-Kimura K, Shimada N, Sogame A, *et al*. (1991). Periodontal condition of pregnant women assessed by CPITN. *J Clin Periodontol* 18:751-754.

Mooney J, Hodge PJ, Kinane DF (2001). Humoral immune response in early-onset periodontitis: influence of smoking. *J Periodontal Res* 36:227-232.

Moss KL, Beck JD, Offenbacher S (2005). Clinical risk factors associated with incidence and progression of periodontal conditions in pregnant women. *J Clin Periodontol* 32:492-498.

Moss KL, Serlo AD, Offenbacher S, Beck JD, Mauriello SM, White RP Jr (2007). The oral and systemic impact of third molar periodontal pathology. *J Oral Maxillofac Surg* 65:1739-1745.

Moss KL, Serlo AD, Offenbacher S, Beck JD, White RP Jr (2008). Third molars and the efficacy of mechanical debridement in reducing pathogen levels in pregnant subjects: a pilot study. *J Oral Maxillofac Surg* 66:1565-1569.

Muramatsu Y, Takaesu Y (1994). Oral health status related to subgingival bacterial flora and sex hormones in saliva during pregnancy. *Bull Tokyo Dent Coll* 35:139–151.

Mäkelä M, Salo T, Uitto VJ, Larjava H (1994). Matrix metalloproteinases (MMP-2 and MMP-9) of the oral cavity: cellular origin and relationship to periodontal status. *J Dent Res* 73:1397-1406.

Nebel D, Bratthall G, Ekblad E, Norderyd O, Nilsson BO (2011). Estrogen regulates DNA synthesis in human gingival epithelial cells displaying strong estrogen receptor β immunoreactivity. *J Periodontal Res* 46:622-628.

Nojima N, Kobayashi M, Shionome M, Takahashi N, Suda T, Hasegawa K (1990). Fibroblastic cells derived from bovine periodontal ligaments have the phenotypes of osteoblasts. *J Periodontal Res* 25:179-185.

Odeberg H, Olsson I (1976). Microbicidal mechanisms of human granulocytes: synergistic effects of granulocyte elastase and myeloperoxidase or chymotrypsin-like cationic protein. *Infect Immun* 14:1276-1283.

Ojanotko-Harri A (1985). Metabolism of progesterone by healthy and inflamed human gingiva in vitro. *J Steroid Biochem* 23:1031-1035.

Ojanotko-Harri AO, Harri MP, Hurttia HM, Sewón LA (1991). Altered tissue metabolism of progesterone in pregnancy gingivitis and granuloma. *J Clin Periodontol* 18:262-266.

O'Neil TC (1979a). Maternal T-lymphocyte response and gingivitis in pregnancy. *J Periodontol* 50:178-184.

O'Neil TC (1979b). Plasma female sex hormone levels and gingivitis in pregnancy. *J Periodontol* 50:279-282.

Page RC, Eke PI (2007). Case definitions for use in population-based surveillance of periodontitis. *J Periodontol* 78(Suppl):1387-1399.

Page RC, Kornman KS (1997). The pathogenesis of human periodontitis: an introduction. *Periodontol 2000* 14:9-11.

Parkar MH, Newman HN, Olsen I (1996). Polymerase chain reaction analysis of oestrogen and androgen receptor expression in human gingival and periodontal tissue. *Arch Oral Biol* 41:979-983.

Paster BJ, Dewhirst FE, Olsen I, Fraser GJ (1994). Phylogeny of *Bacteroides*, *Prevotella*, and *Porphyromonas* spp. and related bacteria. *J Bacteriol* 176:725–732.

Persellin RH, Thoi LL (1979). Human polymorphonuclear leukocyte phagocytosis in pregnancy. Development of inhibition during gestation and recovery in the postpartum period. *Am J Obstet Gynecol* 134:250-255.

Piccinni MP, Giudizi MG, Biagiotti R, Beloni L, Giannarini L, Sampognaro S, *et al*. (1995). Progesterone favors the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones. *J Immunol* 155:128-133.

Pirilä E, Sharabi A, Salo T, Quaranta V, Tu H, Heljasvaara R, *et al*. (2003). Matrix metalloproteinases process the laminin-5 gamma 2-chain and regulate epithelial cell migration. *Biochem Biophys Res Commun* 303:1012-1017.

Preshaw PM, Taylor JJ (2011). Periodontal pathogenesis. In Newman MG, Takei HH, Klokkewold PR & Carranza FA (eds) *Carranza's Clinical Periodontology*, 11th edn. St. Louis, Missouri, Elsevier Saunders: 194–216.

Raber-Durlacher JE, Leene W, Palmer-Bouva CC, Raber J, Abraham-Inpijn L (1993). Experimental gingivitis during pregnancy and post-partum: immunohistochemical aspects. *J Periodontol* 64:211-218.

Raber-Durlacher JE, van Steenbergen TJ, Van der Velden U, de Graaff J, Abraham-Inpijn L (1994). Experimental gingivitis during pregnancy and post-partum: clinical, endocrinological, and microbiological aspects. *J Clin Periodontol* 21:549-558.

Rodríguez E, López R, Paez A, Massó F, Montaño LF (2002). 17Beta-estradiol inhibits the adhesion of

leukocytes in TNF-alpha stimulated human endothelial cells by blocking IL-8 and MCP-1 secretion, but not its transcription. *Life Sci* 71:2181-2193.

Rosling B, Dahlén G, Volpe A, Furuichi Y, Ramberg P, Lindhe J (1997). Effect of triclosan on the subgingival microbiota of periodontitis-susceptible subjects. *J Clin Periodontol* 24:881-887.

Ruiz DR, Romito GA, Dib SA (2011). Periodontal disease in gestational and type 1 diabetes mellitus pregnant women. *Oral Dis* 17:515-521.

Samant A, Malik CP, Chabra SK, Devi PK (1976). Gingivitis and periodontal disease in pregnancy. *J Periodontol* 47:415-418.

Sarlati F, Akhondi N, Jahanbakhsh N (2004). Effect of general health and sociocultural variables on periodontal status of pregnant women. *J Int Acad Periodontol* 6:95-100.

Schroeder HE, Listgarten MA (1997). The gingival tissues: the architecture of periodontal protection. *Periodontol 2000* 13:91-120.

Schätzle M, Löe H, Bürgin W, Anerud A, Boysen H, Lang NP (2003). Clinical course of chronic periodontitis. I. Role of gingivitis. *J Clin Periodontol* 30:887-901.

Scott DA, Krauss J (2012). Neutrophils in periodontal inflammation. *Front Oral Biol* 15:56-83.

Sennström MB, Brauner A, Byström B, Malmström A, Ekman G (2003). Matrix metalloproteinase-8 correlates with the cervical ripening process in humans. *Acta Obstet Gynecol Scand* 82:904-911.

Shah HN, Gharbia SE (1992). Biochemical and chemical studies on strains designated *Prevotella intermedia* and proposal of a new pigmented species, *Prevotella nigrescens* sp. nov. *Int J Syst Bacteriol* 42:542-546.

Shamamian P, Schwartz JD, Pocock BJ, Monea S, Whiting D, Marcus SG, *et al.* (2001). Activation of progelatinase A (MMP-2) by neutrophil elastase, cathepsin G, and proteinase-3: a role for inflammatory cells in tumor invasion and angiogenesis. *J Cell Physiol* 189:197-206.

Shchipkova AY, Nagaraja HN, Kumar PS (2010). Subgingival microbial profiles of smokers with periodontitis. *J Dent Res* 89:1247-1253.

Shu L, Guan SM, Fu SM, Guo T, Cao M, Ding Y (2008). Estrogen modulates cytokine expression in human periodontal ligament cells. *J Dent Res* 87:142-147.

Silness J, Löe H (1964). Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condtion. *Acta Odontol Scand* 22:121-135.

Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. (1998). Microbial complexes in subgingival plaque. *J Clin Periodontol* 25:134-144.

Somerman MJ, Young MF, Foster RA, Moehring JM, Imm G, Sauk JJ (1990). Characteristics of human periodontal ligament cells in vitro. *Arch Oral Biol* 35:241-247.

Sorsa T, Salo T, Koivunen E, Tyynelä J, Konttinen YT, Bergmann U, *et al*. (1997). Activation of type IV procollagenases by human tumor-associated trypsin-2. *J Biol Chem* 272:21067-21074.

Sorsa T, Tjäderhane L, Salo T (2004). Matrix metalloproteinases (MMPs) in oral diseases. *Oral Dis* 10:311-318.

Sorsa T, Tjäderhane L, Konttinen YT, Lauhio A, Salo T, Lee HM, *et al*. (2006). Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Ann Med* 38:306-321.

Sorsa T, Hernandez M, Leppilahti J, Munjal S, Netuschil L, Mäntylä P (2010). Detection of gingival crevicular fluid MMP-8 levels with different laboratory and chair-side methods. *Oral Dis* 16:39-45.

Stabholz A, Soskolne WA, Shapira L (2010). Genetic and environmental risk factors for chronic periodontitis and aggressive periodontitis. *Periodontol 2000* 53:138-153.

Stoker TE, Gibson EK, Zorrilla LM (2010) Triclosan exposure modulates estrogen-dependent responses in the female wistar rat. *Toxicol Sci* 117:45-53.

Stygar D, Westlund P, Eriksson H, Sahlin L (2006). Identification of wild type and variants of oestrogen receptors in polymorphonuclear and mononuclear leucocytes. *Clin Endocrinol (Oxf)* 64:74-81.

Szekeres-Bartho J, Barakonyi A, Par G, Polgar B, Palkovics T, Szereday L (2001). Progesterone as an immunomodulatory molecule. *Int Immunopharmacol* 1:1037-1048.

Söder B, Jin LJ, Wickholm S (2002). Granulocyte elastase, matrix metalloproteinase-8 and prostaglandin E2 in gingival crevicular fluid in matched clinical sites in smokers and non-smokers with persistent periodontitis. *J Clin Periodontol* 29:384-391.

Taani DQ, Habashneh R, Hammad MM, Batieha A (2003). The periodontal status of pregnant women and its relationship with socio-demographic and clinical variables. *J Oral Rehabil* 30:440-445.

Tanner AC, Paster BJ, Lu SC, Kanasi E, Kent R, Jr., Van Dyke T, *et al*. (2006). Subgingival and tongue microbiota during early periodontitis. *J Dent Res* 85:318-323.

Teles R, Sakellari D, Teles F, Konstantinidis A, Kent R, Socransky S, *et al*. (2010). Relationships among gingival crevicular fluid biomarkers, clinical parameters of periodontal disease, and the subgingival microbiota. *J Periodontol* 81:89-98.

Tervahartiala T, Pirilä E, Ceponis A, Maisi P, Salo T, Tuter G, *et al*. (2000). The in vivo expression of the collagenolytic matrix metalloproteinases (MMP-2, -8, -13, and -14) and matrilysin (MMP-7) in adult and localized juvenile periodontitis. *J Dent Res* 79:1969-1977.

Tiililä I (1962). Epulis gravidarum. Thesis. *Proc Finn Dent Soc* 58 (Suppl).

Tilakaratne A, Soory M, Ranasinghe AW, Corea SM, Ekanayake SL, de Silva M (2000). Periodontal disease status during pregnancy and 3 months post-partum, in a rural population of Sri-Lankan women. *J Clin Periodontol* 27:787-792.

Tomar SL, Asma S (2000). Smoking-attributable periodontitis in the United States: findings from NHANES III. National Health and Nutrition Examination Survey. *J Periodontol* 71:743-751.

Tonetti MS, Imboden MA, Lang NP (1998). Neutrophil migration into the gingival sulcus is associated with transepithelial gradients of interleukin-8 and ICAM-1. *J Periodontol* 69:1139-1147.

Tonetti MS, Claffey N (2005). Advances in the progression of periodontitis and proposal of definitions of a periodontitis case and disease progression for use in risk factor research. Group C consensus report of the 5th European Workshop in Periodontology. *J Clin Periodontol* 32 (Suppl):210-213.

Trombone AP, Ferreira SB, Jr., Raimundo FM, de Moura KC, Avila-Campos MJ, Silva JS, *et al*. (2009). Experimental periodontitis in mice selected for maximal or minimal inflammatory reactions: increased inflammatory immune responsiveness drives increased alveolar bone loss without enhancing the control of periodontal infection. *J Periodontal Res* 44:443-451.

Uitto VJ, Suomalainen K, Sorsa T (1990). Salivary collagenase. Origin, characteristics and relationship to periodontal health. *J Periodontal Res* 25:135-142.

Uitto VJ, Nieminen A, Coil J, Hurttia H, Larjava H (1996). Oral fluid elastase as an indicator of periodontal health. *J Clin Periodontol* 23:30-37.

Uitto VJ, Overall CM, McCulloch C (2003). Proteolytic host cell enzymes in gingival crevice fluid. *Periodontol 2000* 31:77-104.

Ujiie Y, Oida S, Gomi K, Arai T, Fukae M (2007). Neutrophil elastase is involved in the initial destruction of human periodontal ligament. *J Periodontal Res* 42:325-330.

Varani J, Perone P, Fligiel SE, Fisher GJ, Voorhees JJ (2002). Inhibition of type I procollagen production in photodamage: correlation between presence of high molecular weight collagen fragments and reduced procollagen synthesis. *J Invest Dermatol* 119:122-129.

Vining RF, McGinley RA, Symons RG (1983). Hormones in saliva: mode of entry and consequent implications for clinical interpretation. *Clin Chem* 29:1752-1756.

Vittek J, Hernandez MR, Wenk EJ, Rappaport SC, Southren AL (1982). Specific estrogen receptors in human gingiva. *J Clin Endocrinol Metab* 54:608-612.

Välimaa H, Savolainen S, Soukka T, Silvoniemi P, Mäkelä S, Kujari H, *et al*. (2004). Estrogen receptor-beta is the predominant estrogen receptor subtype in human oral epithelium and salivary glands. *J Endocrinol* 180:55-62.

Weinberg ED (1987). Pregnancy-associated immune suppression: risks and mechanisms. *Microb Pathog* 3:393-397.

Weiss A, Goldman S, Shalev E (2007). The matrix metalloproteinases (MMPs) in the decidua and fetal membranes. *Front Biosci* 12:649-659.

Wimmer G, Pihlstrom BL (2008). A critical assessment of adverse pregnancy outcome and periodontal disease. *J Clin Periodontol* 35 (8 Suppl):380-397.

Ximenez-Fyvie LA, Haffajee AD, Socransky SS (2000). Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. *J Clin Periodontol* 27:648-657.

Yalcin F, Basegmez C, Isik G, Berber L, Eskinazi E, Soydinc M, *et al*. (2002a). The effects of

periodontal therapy on intracrevicular prostaglandin E2 concentrations and clinical parameters in pregnancy. *J Periodontol* 73:173-177.

Yalcin F, Eskinazi E, Soydinc M, Basegmez C, Issever H, Isik G, *et al*. (2002b). The effect of sociocultural status on periodontal conditions in pregnancy. *J Periodontol* 73:178-182.

Yokoyama M, Hinode D, Masuda K, Yoshioka M, Grenier D (2005). Effect of female sex hormones on *Campylobacter rectus* and human gingival fibroblasts. *Oral Microbiol Immunol* 20:239-243.

Yokoyama M, Hinode D, Yoshioka M, Fukui M, Tanabe S, Grenier D, *et al.* (2008). Relationship between *Campylobacter rectus* and periodontal status during pregnancy. *Oral Microbiol Immunol* 23:55-59.