Periodontitis Preeclampsia

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"Willen moet je ook maar kunnen"

(A. van Dantzig)

Voor mijn 'meiden',
Kimo en Demi

Paranimfen

Drs. E. Hekman
Drs. H.B. van Olm

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List of abbreviations

AUC	Area Under The Curve
BME	2β-Mercaptoethanol
BMI	Body Mass Index
BOP	Bleeding On Probing
BSA	Bovine Serum Albumin
CAL	Clinical Attachment Level
CD	Cluster of Differentiation
CEJ	Cementoenamel Junction
CI	Confidence Interval
CRP	C-reactive Protein
CVD	Cardiovascular Diseases
DAMPs	Damage-associated Molecular Patterns
DPSI	Dutch Periodontal Screening Index
Ec-LPS	*E. coli* Lipopolysaccharide
ET-1	Endothelin-1
FACS	Foetal Calf Serum
G1	Gap 1 (also: Growth 1)
GR	Gingival Recession
IL	Interleukin
IL1RN	Interleukin-1 Receptor Antagonist gene
IUGR	Intra Uterine Growth Restriction
IQR	Interquartile Range
Kg bw	Kilogram body weight
LPS	Lipopolysaccharide
MFI	Mean Fluorescence Intensity
MM6	Mono Mac 6
NLRs	NOD-like Receptors
OR	Odds Ratio
PAMPs	Pathogen-associated Molecular Patterns
PBS	Phosphate-Buffered Saline
PGE	Prostaglandin E
Pg-LPS	*P. gingivalis* Lipopolysaccharide
PISA	Periodontal Inflamed Surface Area
PlGF	Placental Growth Factor
PPD	Pocket Probing Depth
PRR	Pattern Recognition Receptors
RA	Rheumatic Arthritis
RCT	Randomised-controlled Trial
RLRs	RIG-I-like Receptors
RPMI	Complete Roswell Park Memorial Institute
RR	Relative Risk
RT	Room Temperature
RTF	Reduced Transport Fluid
SD	Standard Deviation
sENG	Soluble Endoglin
sFlt-1	Soluble Fms-like tyrosine kinase-1
SNPs	Single-nucleotide Polymorphisms
SRP	Scaling and Root Planing
STBMs	Syncytiotrophoblast Membrane Microparticles
T^H	T-helper
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cells

Bacterial nomenclature

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
E. corrodens	*Eikenella corrodens*
E. coli	*Escherichia coli*
F. nucleatum	*Fusobacterium nucleatum*
P. gingivalis	*Porphyromonas gingivalis*
P. intermedia	*Prevotella intermedia*
P. micra	*Parvimonas micra (formerly Micromonas micros)*
T. forsythia	*Tannerella forsythia*
T. denticola	*Treponema denticola*

CHAPTER 1

General introduction

General introduction

Periodontitis is a microbial inflammatory disease affecting the tooth supporting tissues[1]. Some recent studies showed that periodontitis is associated with adverse pregnancy outcome[2-8]. One of the pregnancy complications suggested to be related with periodontitis is preeclampsia. Preeclampsia is defined by hypertension and proteinuria in the second half of pregnancy and is characterised by systemic vascular dysfunction together with an enhanced inflammatory response[9]. Since periodontitis also is associated with systemic inflammation and endothelial dysfunction[10], this prompted us to explore the possible role of periodontitis in the pathophysiology of preeclampsia, resulting in the clinical and experimental studies, described in this thesis.

In the general introduction, first, periodontal diseases, periodontal pathogens, host-parasite interactions and the reported effects on general health are described. Next, the interaction between periodontal diseases and pregnancy complications are described, with a special focus on preeclampsia, leading to the objectives of the present thesis.

Periodontal diseases

Periodontal diseases are plaque induced infections and pathological changes of the periodontium, traditionally divided into two categories: gingivitis and periodontitis[1]. Gingivitis, the mildest form of periodontal diseases, affects up to 90% of the world wide population and manifests clinically as edema and erythema of the marginal gingiva. Gingivitis does not affect the underlying tooth anchoring structures (connective tissue and alveolar bone) and is readily reversible by effective tooth cleaning and oral hygiene home care[11]. Inflammation that extends deeper into the supportive tissues and involves the periodontal ligament and alveolar bone is known as periodontitis[1]. This apical extension of the inflammatory process results in progressive destruction of tooth anchoring collagen fibers and supportive bone structures, clinically leading to the formation of deepened soft tissue pockets between the gingiva and the surface of the tooth[1]. Moreover, a chronic inflammatory process is instituted with total inflammatory surface areas reaching up to 40 cm^2 on the tooth facing part of the gingiva[12]. Once periodontal pockets are formed and colonised with bacteria, improving oral hygiene alone is no longer sufficient to reverse the inflammatory process. Intensive professional periodontal treatment, including supra- and subgingival scaling and rootplaning (SRP) and surgical pocket elimination is necessary to restore periodontal health[1].

Micro-organisms involved in periodontal diseases

A shift in microbial species in the dental plaque from a relatively harmless gram-positive, facultative anaerobic fermentative population to a predominantly gram-negative, anaerobic and proteolytic virulent population has been strongly associated with periodontal tissue breakdown[13]. Although more than 500 bacterial species have been

identified in the dental plaque[14-17], only a small proportion of these micro-organisms are considered to play a role in the development of periodontitis. Key pathogens associated with periodontitis in adult subjects are the gram-negative micro-organisms *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans), Porphyromonas gingivalis (P. gingivalis), Prevotella intermedia (P. intermedia), Tannerella forsythia (T. forsythia), Fusobacterium nucleatum* (*F. nucleatum*) and the gram-positive *Parvimonas micra (P. micra)*[18]. These bacteria produce a variety of toxins[19-21], such as outer membrane lipopolysaccharides, outer membrane lipids or lipoproteins, peptidoglycan and proteolytic enzymes, which may have detrimental effects on the periodontal tissues[22]. Especially *P. gingivalis*, a black pigmented anaerobic micro-organism, has been reported to possess a variety of virulence factors, including lipopolysaccharide (LPS)[19;20;23]. *P. gingivalis* LPS has the capacity to upregulate the host's immune response[24-26], which may lead to an increased production of local inflammatory mediators and effector molecules including cytokines[27], chemokines[27] and matrix metalloproteinases[28], and finally to periodontal breakdown[28]. Moreover, several serotypes of *P. gingivalis* with varying virulence properties have been described[29-32]. This may suggest that different *P. gingivalis* serotypes with varying inflammatory capacities exist. Alterations in cytokine production after stimulation with different *P. gingivalis* serotypes have indeed been reported[33-36].

Host-parasite interactions

One of the first ways in which the host's immune system recognises different micro-organisms is through pattern recognition receptors (PRR) such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and DNA receptors (cytosolic sensors for DNA) on innate immune cells[37]. TLRs are specific pattern recognition receptors present on a variety of cells, such as monocytes, macrophages and dendritic cells[38], as well as on (oral) epithelial cells[39], gingival fibroblasts[40] and endothelial cells[41]. These TLRs detect so-called damage-associated molecular patterns (DAMPs) [42]. A subtype of DAMPs, pathogen-associated molecular patterns (PAMPs), arise from pathogens and alarm an individual to invading pathogens[42]. Several surface components present on micro-organisms, like LPS, the major component of gram-negative bacterial cell walls, or peptidoglycan, the major component of gram-positive bacterial cell outer membranes are PAMPs and are recognised by TLRs[43;44]. Ligation of TLRs by LPS or peptidoglycan results in activation of the intracellular signalling pathway and ultimately in the production of an array of cytokines[45]. These cytokines modulate inflammatory reactions. At present, 12 mammalian TLRs have been identified[37], of which TLR4 and TLR2 have been studied most frequently[46;47]. TLR4 plays an important role as a receptor for bacterial LPS from gram-negative micro-organisms, while TLR2 mainly recognises peptidoglycan or lipoproteins/lipopeptides from gram-

positive bacteria[48;49]. Activation of TLR4 leads to the production of pro-inflammatory cytokines, like tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6, IL-12. Activation of TLR2 leads to the production of prostaglandin E2 (PGE_2), IL-10 and IL-13, of which in particular IL-10 has been recognised as a potent immunosuppressive and anti-inflammatory molecule[47;50]. Thus, the micro-organisms in the dental plaque, that consists of an array of gram-positive and gram-negative species, may influence the nature of the host response to this polybacterial challenge, by activating specific TLRs[51].

In addition, periodontitis also has a genetic component. Various single-nucleotide polymorphisms (SNPs), for example in the IL-1β, IL-1RN and TLR4 genes, have been associated with chronic and aggressive periodontitis[52]. Thus, not only the mere presence of pathogenic micro-organisms in the biofilm, but also the genetically determined mode of host inflammatory responses to these pathogens determines the clinical manifestations of periodontitis, e.g. the severity of periodontal tissue breakdown[53]. It has indeed been shown that not all individuals are equally affected by the accumulation of dental plaque[54]. Some susceptible individuals may develop aggressive forms of periodontitis at a young age, while others never, or very slowly develop periodontitis[55]. The findings that increased levels of inflammatory mediators, such as TNF-α, IL-1β and PGE_2, correlate with periodontal destruction[56-58] and the fact that these mediators aggravate the inflammatory response[59], has led to the hypothesis that some individuals may be "high-responders" and respond to periodontal infection with high levels of inflammatory mediators which results in periodontal breakdown[54]. Indeed, increased TNF-α and PGE_2 production has been observed in patients with aggressive (early-onset) periodontitis as compared with patients with generalised periodontitis[60]. A significant portion of the host predisposition to periodontitis may therefore relate to excessive innate host defenses and the subsequent activation of the inflammatory response[13].

Systemic effects of periodontal diseases

The local infection and subsequent inflammatory response of the host may not be limited to the periodontal tissues. With daily tooth brushing or even gentle mastication, periodontal micro-organisms or their bacterial products like LPS may enter the blood stream and disseminate into the systemic circulation[61;62]. This chronic or even permanent bacteremia or endotoxemia may activate inflammatory cells and endothelial cells, leading to endothelial cell activation and dysfunction as well as generalised inflammation[63-66], with increased numbers of peripheral blood leukocytes [62] and increased levels of IL-6 and C-reactive protein (CRP)[62;67-69]. Most of these systemic markers of inflammation are also regarded as predictive markers for cardiovascular diseases (CVD) and it is believed that the systemic level of inflammation in periodontitis increases the risk for CVD[70;71]. Indeed, over the last years, meta-analyses

of observational studies showed that patients with periodontitis are at increased risk of developing cardiovascular diseases (CVD)[72-75]. The persistent evidence already has led to a consensus paper on the relationship between CVD and periodontitis, which was published concurrently in two leading journals in their fields, the American Journal of Cardiology (AJC), and the Journal of Periodontology (JOP)[24]. This consensus statement suggests that cardiologists may now examine a patient's mouth, and periodontists may begin asking questions about cardiovascular health and family history of cardiovascular diseases. It was confirmed in a meta-analysis by D'Aiuto et al in 2013 that periodontal treatment reduced CVD biomarkers and improves endothelial function[76]. The importance for periodontal diagnosis and therapy in atherosclerotic individuals to improve their cardiovascular risk profile has been further emphasised upon by Gunupati et al[77], who showed that periodontal treatment reduced biomarkers for atherosclerotic disease in periodontitis patients already suffering from CVD.

Periodontal diseases are also implicated as a risk factor for diabetes mellitus[78] and may be related to a number of other systemic diseases, such as chronic obstructive pulmonary disease (COPD), pneumonia, chronic kidney disease, rheumatoid arthritis (RA), cognitive impairment, obesity, metabolic syndrome and cancer[79;80]. It was shown that treatment of periodontitis in patients with diabetes mellitus leads to an improvement of glycaemic control[81-83], and may reduce RA severity and systemic inflammation in RA patients[84;85].

Periodontal diseases during pregnancy

Periodontal health during pregnancy is another topic that has engendered much interest over the past 50 years. For many years, it has been well established that during pregnancy (in 30-100% of women), a progressive increase in gingival inflammation is observed[86-90]. This phenomenon is referred to as pregnancy gingivitis and disappears post-partum with no permanent effects on the periodontal attachment[88-90]. Although the exact mechanisms remain to be elucidated, it is thought that elevated levels of circulating progesterone or oestrogen during pregnancy contribute to enhanced gingival vascular permeability and enhanced gingival exudates[88;91-94]. This may lead to clinical alterations of the gingiva, i.e. increased redness, oedema and a greater bleeding tendency, which clinically resemble inflammation[95]. Unfortunately, this condition not only leads to greater gingival probing depths[96], but subsequently may transform the gingiva into an environment that may favour the overgrowth of specific bacteria[1]. Indeed, in a recently conducted cohort study, the presence of *P. gingivalis* significantly contributed to the worsening of gingival inflammation during the second and third trimester of pregnancy[97].

Pregnancy gingivitis may also be related to altered immune and inflammatory responses during pregnancy[98;99]. It is evident that during normal pregnancy the

inflammatory response is mildly activated[100]. This activation of the inflammatory response during normal pregnancy is characterised by increased expression of activation markers on monocytes and granulocytes[101-103], differences in monocyte cytokine production[104-107] and increased plasma levels of inflammatory markers, such as CRP[108]. This has led to the concept that pregnancy is a pro-inflammatory condition[109] and might partly explain the increased susceptibility to gingivitis during pregnancy.

Periodontal diseases as a risk factor for pregnancy complications

It was first demonstrated in 1994 in an animal model that the presence of periodontal pathogens might have a negative effect on pregnancy outcome[110]. Collins et al demonstrated that *P. gingivalis*, infused in the pregnant hamster, could elicit low birth weight[110]. In humans, the first evidence of a possible association between periodontitis and adverse pregnancy outcomes was published in 1996 by Offenbacher et al[111], who reported a relationship between periodontitis and preterm low birth weight. Since then, the relationship between periodontitis and complications of pregnancy has become a major topic of research in the field of dentistry. Various observational studies have been carried out, and many systematic reviews support the hypothesis that periodontitis is associated with preterm labour and other conditions complicating pregnancy, such as low birth weight, stillbirth, miscarriage and intrauterine growth retardation[2-8;112]. Perhaps the strongest and most convincing evidence of a relationship between periodontitis and preterm low birth weight until now comes from a meta-analysis published by Vergnes et al[6]. The authors concluded from their data that pregnant women with periodontitis have a 2.8-fold increased risk on preterm birth.

There are several pathophysiological mechanisms by which periodontitis may interfere with pregnancy. A route via the peripheral circulation to the placenta is likely to be involved, since periodontopathic bacteria have been found in human placental tissues[113-116], in amniotic fluid[117;118] or in chorionic tissues[119;120]. The presence of periodontal micro-organisms in foetal and maternal tissues has been shown to induce chorioamnionitis[121], an intrauterine status of inflammation in tissues of either mixed foetal-maternal or foetal origin[122]. Chorioamnionitis is an independent risk factor for preterm birth[123;124] and foetal morbidity[122;125]. Also, the translocation of bacterial products like LPS, and the action of maternally produced inflammatory mediators such as TNF-α and PGE_2 could initiate labour[126]. As PGE_2 mediates cervical ripening and stimulates uterine contractions, it plays a role in the onset of labour[127]. It has been theorised that the increased TNF-α and PGE_2 levels accompanying periodontitis may interfere with normal physiological mechanisms of parturition, resulting in preterm labour[126;128].

If periodontal infections are a cause of preterm birth, it might be expected that eradication of such infections would reduce the risk of preterm birth. Although the

results of initial studies of periodontal treatment to reduce the risk of preterm labour were positive[8], these findings could not consistently be confirmed in later studies[5;129-133]. However, a recently published meta-analysis of RCTs found a significant reduction of preterm birth after periodontal treatment in a subgroup of high risk of preterm birth[134].

Pathogenesis of preeclampsia

Another complication of pregnancy that recently has achieved attention in dental research is preeclampsia. Preeclampsia is a maternal multi-organ disease, clinically manifest during the second half of pregnancy by hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg) with proteinuria (≥300 mg/24 hours or 2+ dipstick), often accompanied with varying dysfunction of major organs as the liver, the kidneys and the brain[135]. It is one of the leading causes of maternal and foetal morbidity and mortality in the western world, with a prevalence of approximately 2-8%[9]. In the Netherlands, preeclampsia is the main cause of maternal mortality, showing a rising trend in the period 1993-2005[136]. Several factors have been implicated in the pathogenesis of preeclampsia, including genetic, immunologic, inflammatory, ethnic, socioeconomic and environmental factors, but until now the exact cause and pathogenesis are not fully clarified.

The current leading hypothesis is that the pathological processes that underlie preeclampsia occur in two stages[9]. Stage one starts with abnormal placentation and impaired vascular remodeling of the myometral spiral arteries that supply the placental bed[137]. This impaired remodeling is most likely the result of deficient trophoblast invasion into the myometrial arteries[138]. The consequence is insufficient placental perfusion in the second half of pregnancy, when the increasing demands of the growing foetus can no longer be met. Subsequently, this leads to placental oxidative stress and the release of various bioactive factors into the maternal circulation[9]. These bioactive factors include inflammatory cytokines such as TNF-α and IL-6[139], as well as syncytiotrophoblast membrane microparticles (STBMs), which may challenge maternal inflammatory cells[140]. They also include antiangiogenic factors such as soluble endoglin (sEng), the soluble form of the vascular endothelial growth factor (VEGF) receptor (sFlt-1) and placental growth factor (PlGF), which may challenge the maternal vascular endothelium[141]. This may lead to stage two, endothelial dysfunction and an excessive inflammatory response leading to the maternal clinical features of the syndrome[9;103;142-145]. Preeclampsia, therefore, is most likely the result of a generalised exacerbation of the inflammatory response, including activation of inflammatory and endothelial cells[109;138;146].

Besides abnormal placentation, other maternal factors predisposing to a pro-inflammatory state, such as obesity, diabetes mellitus and chronic infection, may play a role in the pathogenesis of the syndrome[147].

Periodontal diseases in the pathogenesis of preeclampsia

A possible link between periodontitis and preeclampsia was first proposed in 2003, and since then, a considerable amount of studies focused on this association[148-168]. Although findings were not consistent, there appeared to be a relationship between periodontitis and preeclampsia. One of the mechanisms involved in this putative relationship may be the systemic nature, i.e. the low grade inflammation in periodontitis[62]. It can be hypothesised that the chronic inflammation of periodontitis superimposes on the already pro-inflammatory state of normal pregnancy. The result may be an abnormally activated inflammatory system that ultimately leads to preeclampsia[169;170]. Also a direct bacterial infection of maternal organs by invading periodontopathic bacteria, might explain a possible role of periodontitis in the aetiology of preeclampsia. The involvement of specific periodontal micro-organisms in the pathogenesis of preeclampsia has been examined in some human studies[153;155;171;172]. In these studies, a higher prevalence of *P. gingivalis*[153;171], *T. forsythia*[153], *E. corrodens*[153;171], *P. intermedia*[155] or *A. actinomycetemcomitans*[172] was found in subgingival plaque samples of preeclamptic women as compared with healthy pregnant controls. *P. gingivalis, E. corrodens*, *T. forsythia*, *P. intermedia* and *A. actinomycetemcomitans* all produce a variety of pro-inflammatory factors, including LPS[19;20;173;174], which may affect the immune response during pregnancy. Until now, however, there are no studies to substantiate a causal role of periodontal bacteria or bacterial products in the pathogenesis of the maternal syndrome of preeclampsia. Interestingly, Faas et al[175] established an animal model for preeclampsia, in which an infusion of a low dose of LPS from the enteric bacterium *Escherichia coli* (*E. coli*) into pregnant rats led to activation of the inflammatory response and subsequently to a preeclampsia-like syndrome, characterised by hypertension and proteinuria. This syndrome was pregnancy specific: identically treated non-pregnant rats did not develop these symptoms. Therefore it might be possible that the state of pregnancy also influences the susceptibility for periodontopathic micro-organisms, like *P. gingivalis* or its LPS, leading to increased activation of inflammatory cells and pro-inflammatory cytokine production by leukocytes. This may increase the risk on developing endothelial activation/dysfunction and eventually result in the maternal syndrome of preeclampsia.

Objectives of this thesis

The focus on periodontitis as a risk-factor for preeclampsia, from a scientific point of view, is relatively new. Several research groups in various countries focused on this putative relationship and a range of epidemiological studies with varying results have been published. The first aim was therefore to review the current available literature and to provide an appraisal of the scientific evidence for an association between periodontitis and preeclampsia (*chapter 2*).

Since study outcomes may depend on the adopted definition for periodontitis and preeclampsia, and ethnicity may play a role in the risk on developing both periodontitis as well as preeclampsia[176;177], the second aim of this thesis was to investigate the periodontal condition in a homogenous Caucasian Dutch population with a recent history of early-onset preeclampsia (*chapter 3*).

The third aim was to explore possible causal mechanisms that could explain a role of periodontitis in the pathogenesis of preeclampsia. Although LPS from *P. gingivalis* has been shown to have a negative effect on the developing foetus in animal models in the past[121;178-181], there is no information whether LPS of *P. gingivalis*, like *E. coli* LPS, can induce the maternal symptoms of preeclampsia. An experimental animal study was therefore conducted to examine whether infusion of *P. gingivalis* LPS in pregnant rats, next to affecting foetal growth, also induced the clinical features of preeclampsia characterised by hypertension, proteinuria and generalised systemic inflammation (*chapter 4*).

Pregnant individuals have been shown to be more sensitive to LPS of *E. coli*, as compared with non-pregnant individuals[182]. It is unknown whether during pregnancy the sensitivity to LPS of *P. gingivalis* is also altered and if differences in response may be the consequence of differences in cytokine production. Therefore, the fourth aim was to evaluate cytokine production by leukocytes upon stimulation of whole blood of pregnant and non-pregnant women with bacteria as well as isolated LPS of *P. gingivalis*, with *E. coli* as reference strain *(chapter 5)*.

In addition, since there may be differences in pro-inflammatory properties between different *P. gingivalis* serotypes[33-36], the final aim was to further investigate the host response to *P. gingivalis* and investigate whether variation in pro-inflammatory capacities of its LPS may determine virulence properties of encapsulated and non-encapsulated *P. gingivalis* serotypes[34] *(chapter 6)*.

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CHAPTER 2

Periodontitis and preeclampsia: a systematic review

This chapter is an edited version of the manuscript:
Kunnen A, van Doormaal JJ, Abbas F, Aarnoudse JG, van Pampus MG, Faas MM. Periodontal disease and pre-eclampsia: a systematic review. *J Clin Periodontol* 2010;37:1075–1087

Abstract

Aim: This review evaluates the possible relationship between periodontal disease and preeclampsia, a major pregnancy complication. A generalized inflammatory response plays an important role in the pathogenesis of preeclampsia. Because periodontal disease is a low grade inflammatory state, periodontal disease might contribute to the pathogenesis of preeclampsia.

Main findings and conclusion: A literature search of PubMed, EMBASE and CINAHL until August 2010 revealed 12 eligible observational studies and three randomized-controlled trials (RCTs). It appeared difficult to compare these studies, due to variations in definitions of periodontal disease and preeclampsia, timing of periodontal examination and inadequate control for confounding factors. Eight observational studies reported a positive association, while four studies found no association. None of the RCTs reported reductions in preeclamptic rate after periodontal therapy during pregnancy. Therefore, it is questionable whether periodontal disease plays a causal role in the pathogenesis of preeclampsia. The observed association in eight observational studies might be the result of induction of periodontal disease due to the preeclamptic state or it may be an epiphenomenon of an exaggerated inflammatory response to pregnancy. Larger RCTs with preeclampsia as the primary outcome and pathophysiological studies are required to explore causality and to dissect biological mechanisms involved.

Introduction

Periodontal disease is a chronic destructive inflammatory disease affecting the tooth-supporting tissues and is one of the most prevalent chronic infections in humans. The disease is caused by dental plaque, a biofilm in which gram-negative anaerobic microorganisms dominate. Plaque-associated periodontal diseases can be divided into gingivitis and periodontitis. Gingivitis refers to an inflammatory state of the gums, with no loss of periodontal attachment fibres or alveolar bone. In periodontitis, progressive destruction of collagen fibres and supportive bone structures occurs[1]. Periodontal disease is initiated by oral microorganisms, but it is believed that the severity of periodontal breakdown is orchestrated by the inflammatory response of the host[2].

The inflammatory response may not be limited to the periodontal focus. It has been proposed that daily episodes of bacteremia or dissemination of bacterial endotoxins originating from the periodontal focus may induce systemic activation of the inflammatory response[3;4]. Bacteria or bacterial endotoxins in the systemic circulation may induce pro-inflammatory cytokine production[5]. These cytokines then further activate the inflammatory response, resulting in a chronic low-grade systemic up-regulation of inflammatory responses involving IL-6 and C-reactive protein (CRP)[6-9]. It also includes activation of inflammatory cells and endothelial cells and may result in endothelial dysfunction[10-13].

In pregnancy, the immune response plays a pivotal role in maintaining a healthy equilibrium between mother and foetal allograft[14]. During normal pregnancy, not only the specific immune response is shifted towards a Th2 type immune response but also the inflammatory response is activated[15]. This activation of the inflammatory response during pregnancy is characterized by increased expression of activation markers on monocytes and granulocytes[16], differences in monocyte cytokine production[17], increased circulating levels of pro-inflammatory cytokines[18] and inflammatory markers, such as CRP[19].

It has been suggested that exacerbation of this inflammatory response during pregnancy may result in pregnancy complications, e.g. preeclampsia[15]. Preeclampsia is a maternal multi-organ disease, clinically manifest in the second half of pregnancy by the appearance of hypertension and proteinuria[20]. It is a disorder unique to pregnancy, with a prevalence of approximately 2-3% and it is one of the leading causes of maternal morbidity and mortality in the western world[21]. The pathogenesis is not completely understood, but it is generally accepted that endothelial dysfunction of the maternal vascular system plays a key role in the clinical manifestations of the disease. Preeclampsia is most likely the result of a generalized inflammatory response, including activation of inflammatory and endothelial cells[22-24]. As compared with normal pregnancy, there is increased activation of this inflammatory response during preeclampsia[16;25-28].

Women with diseases associated with chronic low-grade inflammation, such as

diabetes mellitus, hypertension, obesity and arterial diseases are at an increased risk of developing preeclampsia[29-31]. Because periodontal disease is also associated with low-grade inflammation, it can be hypothesized that patients with periodontal disease have an increased risk of developing preeclampsia. A number of studies recently focused on a possible relationship between periodontal disease and preeclampsia. The aim of this review is to evaluate the published scientific evidence for this possible relationship.

Materials and methods

Search strategy

For this review, a thorough search of the literature was performed in the computerized databases of MEDLINE via PubMed (1969-August 2010), EMBASE (1974-August 2010) and CINAHL (2003-August 2010). The search strategy used was a combination of MeSH terms and free text words and is summarized in Table 1.

Table 1. Search strategy

MEDLINE (via PubMed)

("Periodontal Diseases"[MeSH] OR periodont* OR gingivitis) AND ("Pre-Eclampsia"[MeSH] OR preeclampsia OR eclampsia OR eclamp* OR proteinuria OR "pregnancy induced hypertension" OR gestosis OR EPH OR "pregnancy toxemia" OR ("hypertensive disorders of pregnancy") OR "gestational pregnancy" OR "pregnancy-associated hypertension" OR "pregnancy hypertension")
Limits activated: English, French, German, Spanish, Dutch
Run data search: 15 August 2010

EMBASE

'periodontal disease'/exp OR 'periodontal disease' OR periodont* OR 'gingivitis'/exp OR gingivitis AND ('preeclampsia'/exp OR preeclampsia OR 'eclampsia'/exp OR eclampsia OR 'proteinuria'/exp OR proteinuria OR 'pregnancy induced hypertension'/exp OR 'pregnancy induced hypertension' OR eclamp* OR 'gestosis'/exp OR gestosis OR eph OR 'pregnancy toxemia'/exp OR 'pregnancy toxemia' OR 'hypertensive disorders of pregnancy' OR 'gestational pregnancy' OR 'pregnancy-associated hypertension' OR 'pregnancy hypertension'/exp OR 'pregnancy hypertension') AND ([dutch]/lim OR [english]/lim OR [french]/lim OR [german]/lim OR [spanish]/lim)AND [embase]/lim
Run data search: 15 August 2010

CINAHL

S1 Search (MH "Periodontal Diseases+")
S2 Search periodont* OR gingivitis
S3 Search (MH "Pre-Eclampsia+)
S4 Search preeclampsia OR eclampsia OR proteinuria OR "pregnancy induced hypertension" OR gestosis OR EPH OR "pregnancy toxemia" OR "hypertensive disorders of pregnancy" OR "gestational pregnancy" OR "pregnancy-associated hypertension" OR eclamp* OR "pregnancy hypertension"
S5 Search (s1 OR s2) AND (s3 OR s4)
Limiters: peer reviewed; exclude MEDLINE records
Run data search: 15 August 2010

The search was complemented by checking references mentioned in relevant review articles and eligible studies for additional useful publications with the following combinations: "periodontal diseases" and "pregnancy outcomes" or "pregnancy complications" as well as "preeclampsia" and "inflammation" or "infections". The papers were first screened by title and abstract (A.K.). Full-text papers were obtained when the studies fulfilled the criteria of the study selection, as described below. Full-text analysis was performed by two reviewers (A.K., J.v.D.) independently. Case reports, letters, reviews, abstract-only studies and commentaries were excluded from the search.

Inclusion criteria

This systematic review included cross-sectional studies, case-control studies, prospective and retrospective cohort studies and clinical trials. No time restrictions were implemented with respect to the year of publication. The additional inclusion criteria for study selection were:

- Publications in peer reviewed journals on studies in human subjects.
- Papers published in English, French, German, Dutch and Spanish.
- Comparative studies containing original data that evaluated the observational association between periodontal disease and preeclampsia.
- Clinical trials comparing non-surgical periodontal treatment versus no treatment in pregnant women with periodontal disease regarding the rate of preeclampsia.

Quality assessment

Methodological quality was assessed using specific study-design-related checklists based on the quality-assessment forms designed by the Dutch Cochrane Collaboration (Appendix 1, Tables S1-S4). Two reviewers (A.K., J.v.D.) independently generated a score for the articles included. In case of disagreement, a consensus was reached by discussion and if necessary, a third reviewer (F.A.) was consulted. Case-control studies scoring five or more plusses (five out of eight items of the Cochrane checklist for case-control studies) were considered to be methodologically acceptable[32] and were included in this review (Appendix 1, Table S1). For the quality assessment of cohort studies, the Dutch Cochrane checklist for cohort studies was used. Cohort studies scoring six or more plusses (six out of nine items of the Cochrane checklists for cohort studies) were considered methodologically acceptable (Appendix 1, Table S2). Because there was no adequate checklist for cross-sectional studies at the Dutch Cochrane Collaboration, apart from a checklist for diagnostic tests, a quality-assessment checklist had to be developed. This checklist was adapted from the quality checklist used for cohort studies and included items of checklists for cross-sectional studies (diagnostic tests). Cross-sectional studies scoring five or more plusses (out of eight items of this checklist) were included in this review (Appendix 1, Table S3). For clinical trials, studies

scoring six or more plusses (six out of nine items of the Cochrane checklists for randomized-controlled trials) were considered methodologically acceptable (Appendix 1, Table S4). Information of the quality assessment on the studies considered for inclusion is available in the online version of this article (Appendix 1, Tables S1-S4).

Data extraction and synthesis

For each study, the following data were independently extracted by the two observers and recorded in a data sheet:

- Patient selection (patients and controls adequately defined).
- Sample size.
- Selection bias.
- Exposure (periodontal disease).
- Outcome (preeclampsia).
- Blinding of investigators (to pregnancy-outcome).
- Adjustment for well-known confounding factors [age, ethnicity, parity, multiple gestation pregnancies, diabetes mellitus, body mass index (BMI) or chronic hypertension].
- Reported odds ratios (OR) or risk ratios (RR) with 95% confidence interval (CI).
- Follow-up (in case of cohort studies and clinical trials).

Statistical analysis

Because of the high level of clinical heterogeneity in periodontal disease definitions, it was not possible to apply statistical methods to estimate overall pooled risks of periodontal disease from the selected observational studies. For the meta-analyis of the RCTs, the statistical software package 'Stata' was used (STATA 10.0 SE; Stata Corp., College Station, TX). Weighted rates together with random effects models were used to calculate the overall effects for the RCTs included. The Cochran's *Q* test was used to test the homogeneity of the estimates of OR between studies.

Results

Search results

Following the described search strategy, we found 331 articles. The reference lists of these papers and relevant reviews resulted in two additional papers. The screening by title and abstract resulted in 30 eligible articles (Fig. 1).

After full-text reading of these articles, four studies were excluded because they did not investigate the association between periodontal disease and preeclampsia[33-36] and also four pathophysiological studies were excluded[37-40]. Furthermore, one prospective study[41] was excluded because the identical study population had been used in a more recent study[42] and two more studies[43;44] that performed secondary analyses of the same

population. One case-control study[45] was excluded because the population had been added to the sample of a later study[46]. A total of 18 studies fulfilled the inclusion and exclusion criteria and were assessed methodologically.

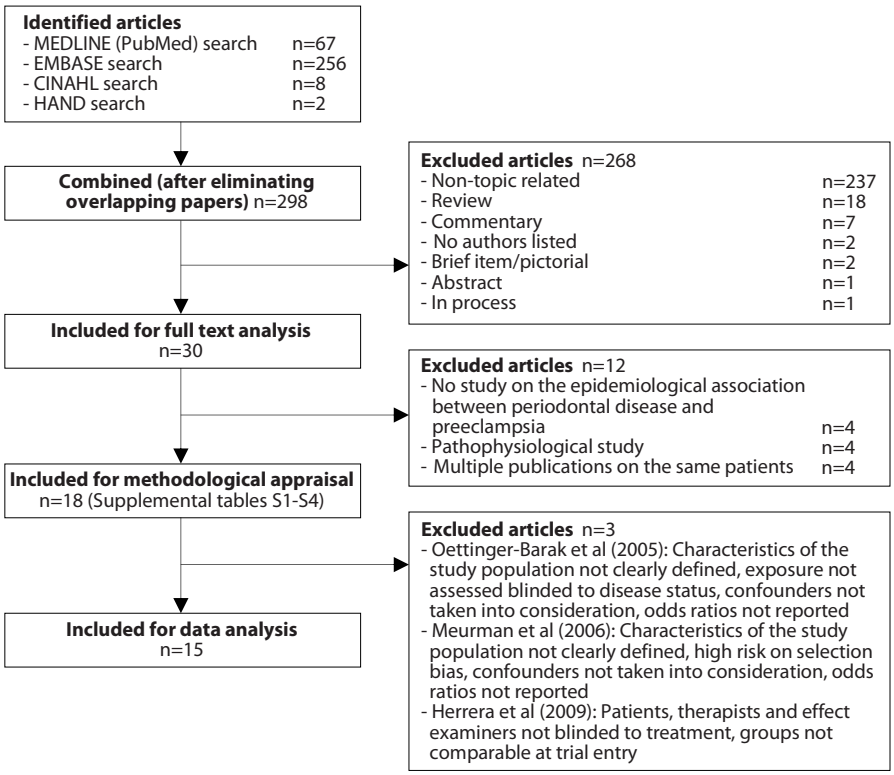

Fig. 1. Flow-chart outlining the search strategy and results along the various steps.

Assessment of quality

Two observational studies[47;48] were excluded from further analysis, because more than three items of the quality assessment checklist scored negative (Appendix 1, Tables S1-S3). Of the 12 observational studies included for further data analyses, three studies[46;49;50] scored positive for all assessed items and were considered to have the highest level of quality with an estimated low risk of bias. Selection bias could not be excluded in six studies[51-56]. Of these, one study also provided insufficient information about study group characteristics[52]. Selective loss-to-follow-up could not be excluded in two of the cohort studies[42;56]. Blinding to outcome has not been reported in four studies[42;53-55], and has not clearly been described in two studies[51;57]. Two studies[52;53] did not adjust for important confounders with respect to the analyses. Two studies did not

clearly define preeclampsia[51;58], while Shetty et al[55] did not clearly specify the adopted periodontal disease definition in their statistical analysis.

One RCT[59] was excluded from further analyses, because more than three items of the quality assessment scored negative (Appendix 1, Table S4). All three RCTs included were largely comparable in methodological quality. Clearly, blinding of patients and dental therapists to treatment was impossible, since treatment consisted of manual periodontal therapy. Two studies[60;61] did not clearly specify whether staff members who included patients were blinded to randomization order.

A total of 15 articles fulfilled the inclusion criteria and passed the quality assessment. All studies appeared to be published in peer-reviewed journals between January 2003 and August 2010. Below, each study is described and summarized in Tables 2 and 3.

Cross-sectional studies

Castaldi et al[52] examined the periodontal condition of 1562 women within 48 h after delivery. Of the total population included, 157 (10%) women were diagnosed with preeclampsia. The periodontal investigators were blinded for pregnancy outcomes. Gingivitis was found in 34.3% and severe periodontal disease in 17.5% of the women. After adjusting for smoking during pregnancy, no association was found between either gingivitis or severe periodontal disease and preeclampsia. The authors did not report to have adjusted for other confounding factors.

Case-control studies

In a matched case-control study, Canakci et al (Canakci et al. 2004) examined within 48 h prior to delivery, the periodontal condition of 41 preeclamptic women and 41 normotensive healthy pregnant women. Cases and controls were individually matched for age, parity, gravidity, smoking and prenatal care. Periodontal disease was present in 46.3% of women with preeclampsia and in 21.9% of controls. After adjusting for serum cholesterol levels, serum triglycerides levels and maternal body weight, the conditional multiple logistic regression analysis showed that preeclampsia was associated with periodontal disease.

Contreras et al[53] carried out a case-control study that included 130 pregnant women with preeclampsia and 243 healthy pregnant women. In both groups the periodontal status was determined between 26 and 36 weeks of pregnancy. Chronic periodontal disease was significantly more prevalent in the preeclamptic group (63.8%) compared with the control group (36.6%). When periodontal disease was further stratified in severity, incipient periodontal destruction was present in 42.3% of cases, compared with 27.2% of controls, whereas moderate-to-severe periodontal destruction was observed in 21.5% of cases, compared with 9.5% of controls. The authors did not report to have adjusted for confounding factors.

Khader et al[49] conducted a blinded case-control study amongst 115 preeclamptic women and 230 healthy controls within 24 h after delivery. Only insured, non-smoking and non-alcohol drinking women were included in this study. After confounding for maternal age, parity, (family) history of preeclampsia, family history of cardiovascular disease, pre-pregnancy BMI, twin birth and self-reported emotional stress during pregnancy, no statistical differences were found between cases and controls concerning any of the eight periodontal parameters investigated.

The association between periodontal disease and early-onset preeclampsia (<34 weeks of pregnancy) was tested in a case-control study conducted by our own group[54]. The periodontal condition of 17 early-onset preeclamptic women and 35 women with uncomplicated pregnancies was examined in a period of 3-28 months postpartum. Severe periodontal disease was present in 82% of the post-preeclamptic women and in 37% of the controls. After adjusting for age, BMI, smoking and educational level, severe periodontal disease was associated with preeclampsia.

The aim of a case-control study performed by Canakci et al[51] was to correlate the severity of periodontal disease to the severity of preeclampsia. Dental and periodontal examinations were performed in 20 mild preeclamptic, 18 severe preeclamptic and 21 healthy pregnant women within 48 h preceding delivery. Mild periodontal disease was found in 16.7% of the severe preeclamptic women, in 25% of the mild preeclamptic women and in 28.6% of the controls. Severe periodontal disease was detected in 72.2% of the severe preeclamptic women, in 50% of the mild preeclamptic women and in 33.3% of the controls. After adjusting for age, smoking, body weight, socioeconomic status and educational level, the results showed that severe periodontal disease was associated with both mild and severe preeclampsia.

Siqueira et al[46] performed a matched case-control study on 125 preeclamptic women and 375 healthy controls within 48 h after delivery. The frequency of periodontal disease before matching was significantly higher among the preeclamptic women (56.7%) than among the control group (39%). After matching and adjusting for maternal age ≥30 years, chronic hypertension, primiparity, previous pre-term birth and prenatal visits, periodontal disease remained associated with preeclampsia. However, when periodontal probing depth (PPD) and clinical attachment level (CAL) were tested with cut-off points of ≥5 mm or ≥7 mm, the ORs for preeclampsia were not significant, indicating that periodontal breakdown in itself was not associated with preeclampsia in this study.

A recent epidemiological study was conducted by Lohsoonthorn et al[50], who examined the periodontal condition of 150 preeclamptic and 150 healthy controls within 48 h after delivery. In the preeclamptic group, 49.3% of the women had mild periodontitis, 21.3% had moderate periodontitis and severe periodontitis was present in 8.0% of the women. In the control group, 54.0% of the women were diagnosed with

mild periodontitis, while moderate periodontitis was present in 20.7% and severe periodontitis in 4.7% of the women. After adjusting for maternal age, educational attainment, parity, pre-pregnancy BMI, annual household income, employment during pregnancy, marital status, onset of pre-natal care, alcohol use and smoking during pregnancy, no association between periodontal disease and preeclampsia was found. In addition to their periodontal disease definition[62], the authors evaluated whether the previously found associations between periodontal disease and preeclampsia were due to differences in adopted periodontal disease definitions. Therefore they used definitions used by Canakci et al[57], Contreras et al[53], Lopez et al[63] and Boggess et al[42] on their dataset. In this Thai population, none of the definitions used by the other authors showed a significant association between periodontal disease and preeclampsia.

In a study conducted by Nabet et al[58], the association between periodontitis and pre-term delivery (<37 weeks of gestation) was analysed according to the causes of pre-term birth. For this purpose, 1108 cases with pre-term delivery (liveborn child between 22-36 weeks of gestation) and 1094 controls with deliveries at term (≥37 weeks of gestation) were included in the study. One hundred and ninety-eight (18.1%) of the cases were induced pre-term deliveries due to preeclampsia. Periodontal examinations were performed within 2-4 days after delivery. Localized periodontitis was found in 13.6% of the cases and in 10.8% of the controls, while generalized periodontitis was present in 20.7% of the cases and in 10.8% of the controls. After adjusting for maternal age, parity, nationality, educational level, marital status, employment during pregnancy, BMI before pregnancy and smoking status, an association was observed between generalized periodontitis and induced pre-term birth due to preeclampsia.

The most recently conducted case-control study so far was conducted by Shetty et al[55], who examined the periodontal condition of 30 preeclamptic women and 100 healthy pregnant controls at recruitment (26-32 weeks of gestation) and within 48 h after delivery. At enrolment, 100% of the cases and 78% of the controls were diagnosed with periodontal disease (CAL ≥3 mm). After adjusting for maternal age, body weight, occupation, education and income, severe periodontal disease (CAL >5 mm) both at enrolment as well as at delivery was associated with an increased risk of preeclampsia. There were no significant differences in disease progression between cases and controls.

Cohort studies

As part of a prospective cohort study on the effect of maternal periodontal disease on obstetric outcomes (the Oral Conditions and Pregnancy Study, OCAP)[41], Boggess et al[42] examined the periodontal condition of 850 pregnant women at enrolment (before 26 weeks' gestation) and followed them until delivery. Thirty-nine women (4.6%) developed preeclampsia. At enrolment, 58.4% of the women were diagnosed with mild

periodontitis and 14.7% had severe disease. In order to determine periodontal disease progression, a second periodontal examination was performed in 763 of the women within 48 h after delivery. At this time, 37.3% of the women had mild periodontal disease and 13.1% had severe periodontal disease. Periodontal disease progression occurred in 26.6% of the women. After adjusting for maternal age, race, insurance status, delivery at <37 weeks' gestation and smoking during pregnancy, the authors found that women were at a higher risk of developing preeclampsia if they had severe periodontal disease at delivery or periodontal disease progression during pregnancy. Periodontal disease at enrolment, however, was not associated with an increased risk of developing preeclampsia.

As part of a large multi-centre prospective cohort study (the Periodontal Infection and Prematurity Study, PIPS), the risk of adverse pregnancy outcomes in women with periodontal disease compared with those without disease was assessed by Srinivas et al[56]. In this study, 311 pregnant women with periodontal disease between 6 and 20 weeks of gestation and 475 without periodontal disease were included. Periodontal examinations were performed by trained nurses. Sixteen women (5.2%) with periodontal disease developed preeclampsia, while in the periodontally healthy group 32 women (6.7%) developed preeclampsia. After adjusting for maternal age, race, tobacco, obesity and chronic hypertension, the authors found no association between the presence of periodontal disease during pregnancy and preeclampsia.

RCTs

In an intervention study conducted by Michalowicz et al[60], the effect of periodontal treatment on pregnancy outcomes was examined. Eight hundred and twelve pregnant women with periodontal disease were randomly assigned to two groups between 13 and 17 weeks of gestation. Periodontal disease was assessed at trial entry, at 21-24 weeks' gestation and at 29-32 weeks' gestation. The treatment group received periodontal therapy before 21 weeks of gestation, which consisted of up to four sessions of scaling and rootplaning (SRP). Treatment participants also received monthly tooth polishing, oral hygiene instruction and if needed, SRP was provided to the treatment group until delivery. The control group received only a brief examination at monthly follow-ups and received periodontal treatment after delivery. Although the primary outcome of this study was gestational age at the end of pregnancy (pre-term birth), the authors also evaluated preeclampsia as one of the secondary outcomes. Periodontal treatment during pregnancy did not significantly alter the rate of preeclampsia, despite the improvement of the periodontal status.

Offenbacher et al[61] also studied the effects of periodontal treatment on the frequency of pre-term birth as the primary outcome and preeclampsia as one of the secondary outcomes, in a randomized treated-masked controlled trial [The Maternal

Oral Therapy to Reduce Obstetric Risk Study (MOTOR)]. One thousand seven hundred and sixty eligible pregnant women with at least 20 teeth present and diagnosed with periodontal disease were randomly assigned to receive periodontal treatment either before 23 6/7 weeks of gestation (treatment group) or after delivery (control group). Periodontal examinations were performed at baseline and at delivery. Periodontal treatment included up to four sessions of SRP, full-mouth tooth polishing and oral hygiene instruction. There were no significant differences in the frequency of preeclamptic pregnancies when comparing the women in the treatment group with those in the control group.

Newnham et al[64], investigated whether periodontal treatment prevented major pregnancy complications, with pre-term birth as the primary outcome, and low birth weight and preeclampsia as secondary outcomes. For this purpose, 1078 pregnant women were allocated at random to receive periodontal treatment at ±20 weeks of gestation (treatment group) or after delivery (control group). Periodontal examinations were performed at trial entry (between 12 and 20 weeks gestation). Periodontal treatment included up to three sessions of SRP, removing of overhanging restorations and comprehensive oral hygiene instructions. Patients were advised to additionally rinse with 0.12% chlorhexidin. At gestational week 28, further examinations were performed and a 3-week follow-up periodontal treatment was offered to the treatment group if necessary, and monthly motivation and oral hygiene instruction was provided. Periodontal examinations were repeated at 32 and 36 weeks' gestation. Although periodontal treatment during pregnancy significantly improved the periodontal status, it did not affect the rate of preeclamptic pregnancies.

Table 2. Overview of the selected observational studies and their characteristics (Level of evidence III-2*)

Study	N	Study design	Race/Ethnicity	Definition of preeclampsia	Definition of periodontal disease	Power analysis or sample size estimation	Conclusions
Boggess et al (2003) USA	850 (763)	Cohort	Black: 47% Caucasian: 48% Other: 5%	RR >140/90 mmHg on 2 occasions and dipstick +1 proteinuria	Moderate periodontal disease: 1-15 sites with PPD ≥4 mm+BOP Severe periodontal disease: ≥15 sites with PPD ≥4 mm+BOP Disease progression: ≥4 sites that increased ≥2 mm in pocket depth	No	Periodontal disease at delivery, but not at enrolment, is associated with an increased risk of preeclampsia: OR 2.4 (1.1-5.3) Disease progression during pregnancy is associated with an increased risk of preeclampsia: OR 2.1 (1.0-4.4)
Canakci et al (2004) Turkey	Ca: 41 Co: 41	Case-control	Turkish 100%	RR ≥140/90 mmHg on ≥2 occasions and ≥300 mg/24h or dipstick +1 proteinuria after 20 weeks' gestation	≥4 teeth with ≥1 sites with PPD ≥4 mm+BOP and CAL ≥3 mm at the same site	No	Maternal periodontal disease during pregnancy is associated with an increased risk of preeclampsia: OR 3.47 (1.07-11.95)
Contreras et al (2006) Colombia	Ca: 130 Co: 243	Case-control	Mixed ethnic: 81% Black: 15% Native: 4%	RR ≥140/90 mmHg and ≥300 mg/24h or dipstick +2 proteinuria	Chronic periodontal disease: ≥2 sites with PPD ≥4 mm, CAL ≥4 mm+BOP Further stratified: - Incipient: CAL 4-5 mm - Moderate/severe: CAL ≥6 mm	Yes	Chronic periodontal disease is associated with preeclampsia: OR 3.0 (1.91-4.87) Association after further stratification: incipient periodontal disease and preeclampsia: OR 2.34 (1.47-3.71), moderate/severe periodontal disease and preeclampsia: OR 3.32 (1.79-6.15)
Castaldi et al (2006) Argentina	1562	Cross-sectional	Not specified	RR ≥140/90 mmHg and >30 mg/dl proteinuria	Gingivitis: >25% BOP and gingival inflammation Severe periodontal disease: ≥4 teeth with ≥1 sites with CAL ≥3 mm	No	No association between either gingivitis and preeclampsia: OR 0.99 (0.69-1.43) or severe periodontal disease and preeclampsia: OR 1.09 (0.67-1.75)
Khader et al (2006) Jordan	Ca: 115 Co: 230	Case-control	Jordanian 100%	RR ≥140/90 mmHg and dipstick +1 proteinuria after 20 weeks' gestation	No definition specified (results based on periodontal parameters)	Yes	No significant differences in periodontal parameters between the preeclamptic group and controls
Kunnen et al (2007) The Netherlands	Ca: 17 Co: 35	Case-control	Caucasian Dutch 100%	Diastolic blood pressure ≥90 mmHg on 2 occasions after 20 weeks' gestation and ≥300 mg/24h or dipstick +2 proteinuria occurring <34 weeks of pregnancy (early-onset)	Moderate periodontal disease: 1-15 sites with PPD ≥4 mm+BOP Severe periodontal disease: ≥15 sites with PPD ≥4 mm+BOP	No	Severe periodontal disease is associated with an increased risk of early-onset preeclampsia: OR 7.9 (1.9-32.8)

*Levels of evidence based on the classification of the National Health and Medical Research Council (NHMRC).
Ca, case group; Co, control group; RR, blood pressure; PPD, pocket probing depth; BOP, bleeding on probing; CAL, clinical attachment level; OR, odds ratio.

Table 2. (Contd.)

Study	N	Study design	Race/Ethnicity	Definition of preeclampsia	Definition of periodontal disease	Power analysis or sample size estimation	Conclusions
Canakci et al (2007a) Turkey	Ca: 38 Co: 21	Case-control	Not specified	Mild preeclampsia: RR ≥140/90 mmHg on ≥2 occasions with or without proteinuria. Severe preeclampsia: RR ≥160/110 mmHg on ≥2 occasions and ≥5g/24h or dipstick ≥3+ proteinuria	Moderate periodontal disease: 1-15 sites with PPD ≥4 mm+BOP Severe periodontal disease: ≥15 sites with PPD ≥4 mm+BOP	No	Both mild and severe preeclampsia are associated with severe periodontal disease: mild preeclampsia: OR 2.43 (1.13-8.19), severe preeclampsia: OR 3.78 (1.77-12.74)
Siqueira et al (2008) Brazil	Ca: 125 Co: 375	Case-control	Multi ethnic	RR >140/90 mmHg on 2 occasions and dipstick ≥+1 proteinuria after 20 weeks' gestation	≥4 teeth with PPD ≥4 mm and CAL ≥3 mm at the same site	No	Maternal periodontitis is associated with preeclampsia: OR 1.52 (1.01-2.29)
Lohsoonthorn et al (2009) Thailand	Ca: 150 Co: 150	Case-control	Thai 100%	RR ≥140/90 mmHg ≥6h apart and ≥300 mg/24h or dipstick +1 on ≥2 specimens ≥4h apart	Mild periodontitis: ≥1 teeth with inter-proximal sites with CAL ≥4 mm and PPD ≥4 mm Moderate periodontitis: ≥2 nonadjacent teeth with inter-proximal sites with CAL ≥5 mm and PPD ≥4 mm Severe periodontitis: ≥2 nonadjacent teeth with inter-proximal sites with CAL ≥6 mm and PPD ≥4 mm	Yes	No significant differences in periodontal parameters between the preeclamptic group and controls; periodontal disease is not associated with an increased risk of preeclampsia: mild periodontal disease and preeclampsia: OR 0.83 (0.43-1.60), moderate periodontal disease and preeclampsia: OR 0.77 (0.35-1.69), severe periodontal disease and preeclampsia: OR 0.92 (0.26-3.28)
Srinivas et al (2009) USA	786	Cohort	Black: 81% Other: 19%	RR ≥140/90 mmHg and ≥300 mg/24h or dipstick +1 proteinuria after 20 weeks' gestation	≥3 teeth with CAL ≥3 mm	Yes	Maternal periodontal disease during pregnancy is not associated with an increased risk of preeclampsia: OR 0.71 (0.37-1.36%)
Nabet et al (2010) France	Ca: 198 Co: 1094	Case-control	Not specified	Systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg with proteinuria 300 mg/24h	Localized periodontitis: 2 or 3 teeth with PPD ≥4 mm and CAL ≥3 mm at the same site Generalised periodontitis: ≥4 teeth with PPD ≥4 mm and CAL ≥3 mm at the same site	Yes	Generalized maternal periodontitis is associated with induced preterm birth due to preeclampsia: OR 2.46 (1.58-3.83) Localized maternal periodontitis is not associated with induced preterm birth due to preeclampsia: OR 1.49 (0.91-2.44)

*Levels of evidence based on the classification of the National Health and Medical Research Council (NHMRC).
Ca, case group; Co, control group; RR, blood pressure; PPD, pocket probing depth; BOP, bleeding on probing; CAL, clinical attachment level; OR, odds ratio.

Table 2. (Contd.)

Study	N	Study design	Race/Ethnicity	Definition of preeclampsia	Definition of periodontal disease	Power analysis or sample size estimation	Conclusions
Shetty et al (2010) India	Ca: 30 Co: 100	Case-control	Indian 100%	Systolic blood pressure ≥140 mmHg or diastolic on ≥2 occasions 4h apart and dipstick ≥+1 proteinuria	Periodontitis: PPD ≥4 mm and CAL ≥3 mm according the Ramfjord Index (16, 22, 24, 36, 42 and 44) at 4 sites per tooth Disease progression: increase in severity and CAL ≥3 mm	No	Maternal periodontitis both at enrolmen [OR 5.78 (2.41-13.89)] as well as at delivery [OR 20.15 (4.55-89.29)] is associated with preeclampsia

*Levels of evidence based on the classification of the National Health and Medical Research Council (NHMRC).
Ca, case group; Co, control group; RR, blood pressure; PPD, pocket probing depth; BOP, bleeding on probing; CAL, clinical attachment level; OR, odds ratio.

Table 3. Overview of the selected randomized-controlled trials and their characteristics (Level of evidence II*)

Study	N	Race/Ethnicity	Definition of preeclampsia	Definition of periodontal disease	Gestational age (wks) at treatment start	Evaluation of effectiveness of therapy	Rate of preeclampsia	Power analysis or sample size estimation	Conclusions
Michalowicz et al (2006) USA	I: 407 Co: 405	Not clearly specified	Pregnancy associated hypertension occurring 4h-14 days after an episode of pregnancy-associated proteinuria	≥4 teeth with PPD ≥4 mm and CAL ≥2 mm as well as BOP ≥35%	<21 wks	Yes	I: 31 (7.6%) Co: 20 (4.9%)	Yes	Periodontal therapy did not reduce the incidence of preeclampsia RR 1.54 (0.89-2.66)
Offenbacher et al (2009) USA	I: 882 Co: 878	Black: 37.6% Caucasian: 61.0% Other: 1.4%	Not reported	≥3 sites with CAL ≥3 mm	<23 6/7 wks	Yes	I: 67 (7.6%) Co: 74 (8.4%)	Yes	Periodontal therapy did not reduce the incidence of preeclampsia RR 0.90 (0.66-1.24)
Newnham et al (2009) Australia	I: 538 Co: 540	Black: 3.7% Caucasian: 73.6% Asian: 16.2% Hispanic: 1.1% Other: 5.3%	Not reported	≥12 sites with PPD ≥4 mm	± 20 wks	Not reported	I: 18 (3.4%) Co: 22 (4.1%)	Yes	Periodontal therapy did not reduce the incidence of preeclampsia RR 0.82 (0.45-1.51)

*Levels of evidence based on the classification of the National Health and Medical Research Council (NHMRC).
I, intervention group; Co, control group; RR, blood pressure; PPD, pocket probing depth; BOP, bleeding on probing; CAL, clinical attachment level; RR, risk ratio.

Meta-analysis of RCTs

After combining the data from the RCTs, the pooled RR of preeclampsia was 1.0 (95% CI 0.78-1.28) (Fig. 2). Statistical heterogeneity is Cochran's Q= 3.24 (df=2, p=0.198).

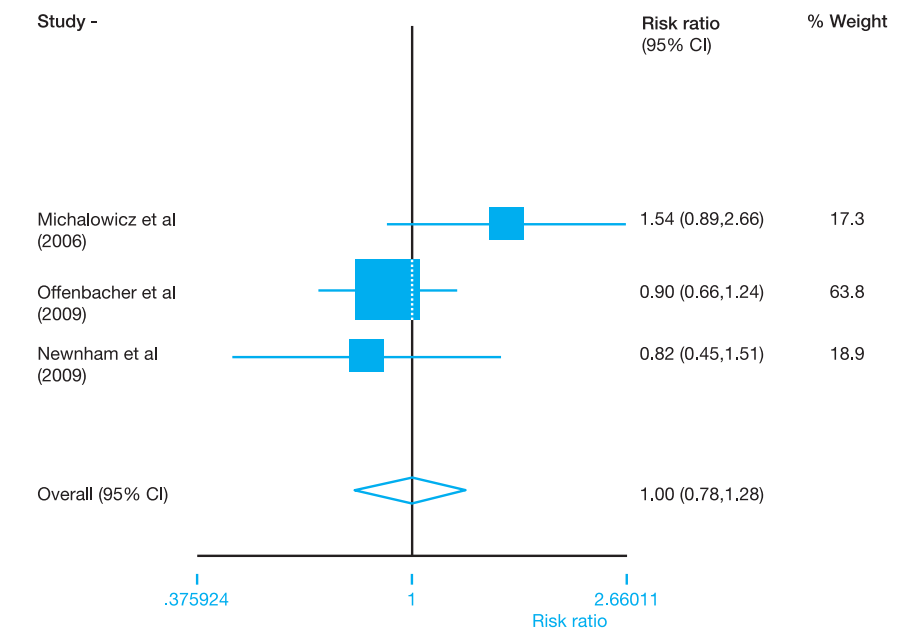

Fig. 2. Fixed effect analysis of the meta-analyses of randomized-controlled trials.

Discussion

The present review summarizes the results of observational studies and RCTs investigating the relationship between periodontal disease and preeclampsia. It shows that an association between periodontal disease and preeclampsia was seen in eight observational studies while four of the observational studies failed to find such an association. None of the RCTs showed a reduction in preeclamptic pregnancies after periodontal treatment during pregnancy.

The reasons for the heterogeneity in findings among the observational studies cannot precisely be determined, because several methodological differences may have biased study outcomes. To be able to assess the methodological quality of the studies, we used specific study-design-related forms based on the Dutch Cochrane Collaboration checklists. Although all studies fulfilled the minimum score of our quality assessment, differences in quality between the studies may contribute to the heterogeneity in results. However, even within the three case-control studies with the

highest methodological quality (plusses on all items), no homogeneity was observed between the results: Khader et al[49] and Lohsoonthorn et al[50] failed to find an association, while Siqueira et al[46] reported a positive association between periodontal disease and preeclampsia. Moreover, although both cohort studies scored seven plusses (out of nine items) on the quality assessment, one cohort study[42] reported a positive association, while the other cohort study [56] found no association between the two diseases. Thus, the observed heterogeneity cannot be explained by methodological quality alone, as there appears to be no relation between the quality of the studies included and their results.

One of the pertinent issues is the potential lack of power calculation, as only five out of 12 observational studies reported to have performed power calculations. This problem can be circumvented by performing a meta-analysis. However, we were not able to do this due to the methodological differences among the observational studies, which is the most obvious limitation of this review. One other important limitation of this review is the variety in clinical disease definitions for periodontal disease. At least nine different periodontal disease definitions were adopted throughout the 15 studies reviewed. Because the strength of the association between periodontal disease and pregnancy outcomes may depend upon the periodontal disease definition[65], it is difficult to compare study outcomes.

Moreover, although commonly accepted periodontal definitions take mean PPD and CAL (or specific cut-off points for PPD and CAL) as a means to classify periodontitis, PPD and CAL are linear measures that do not necessarily reflect active periodontal disease and do not quantify the total inflammatory burden[66]. In order to estimate active periodontal disease, also bleeding on probing (BOP) should be included in the periodontal assessment. It is important to observe that all studies that included BOP in their periodontal disease definition found an association between periodontal disease and preeclampsia[42;51;53;54;57]. In contrast, four out of seven studies that did not include BOP in their definition failed to find an association[49;50;52;56]. Therefore, the adopted criteria for defining periodontal disease in the studies reviewed may not provide the proper tool to draw a decisive conclusion on periodontitis as a risk factor for preeclampsia. Furthermore, eight of the observational studies excluded third molar periodontal pathology from the analysis[46;49-51;54;55;57;58]. It is possible that exclusion of third molar pathology, which has recently been associated with pre-term birth[67] has led to an underestimation of periodontal disease and therefore may have biased study outcomes.

Periodontal disease as well as preeclampsia are of a multifactorial nature, and both diseases share some common risk factors, like ethnicity[68;69]. Although periodontal disease was associated with preeclampsia in certain populations (i.e. Turkish, Dutch and Indian)[54;55;57], it was not in others[49;50], irrespective of the adopted periodontal disease

definition. Thus, in certain ethnic populations, other factors, e.g. genetically determined host responses, might be involved. Some of the studies did not clearly report the ethnicity of their populations[51;52;58], or included heterogeneous populations[42;46;53;56]. Also, smoking and obesity may influence the severity of both conditions[70-74]. Smoking increases the risk of periodontal disease[75], but paradoxically decreases the risk of preeclampsia[76-78]. Although most studies reported to have controlled for smoking or excluded smoking during pregnancy, smoking behaviour was only reported dichotomously (yes/no). Therefore, smoking behaviour may have had some residual confounding effects on the strength of the association. Furthermore, three of the studies did not report or adjust for BMI in their analyses[46;52;53].

All observational studies met the minimum criteria for defining preeclampsia[79]. However, a distinction can be made between early-onset preeclampsia (gestational age <34 weeks) and late-onset preeclampsia (gestational age >34 weeks)[80;81]. The two forms differ in pathogenesis, genetic risk and inheritance[30;81]. Indeed, in the study of Shetty et al[55], a remarkably high incidence of periodontal disease was observed in early-onset preeclamptic women. This could indicate that an association between periodontal disease and preeclampsia may only be evident in certain forms of preeclampsia. Concerning the RCTs, none of the studies reported a clear definition for preeclampsia. Because Offenbacher et al[61] performed therapy up to 24 weeks' gestation, it cannot be excluded that some of the women assigned to the treatment group already developed early-onset preeclampsia before intervention. This may have biased study outcomes.

Inconsistent findings in the observational studies may also be due to differences in the time points of the periodontal screening. The periodontal disease status was examined at different time points during pregnancy or post-partum, ranging from before 26 weeks of gestation to 3-28 months postpartum. The long time span between outcome and exposure in the study of Kunnen et al[54] may have allowed for changes in the periodontal condition. Moreover, one may argue that in this study, preeclampsia was the exposure and the periodontal status the outcome. Therefore, this study may suggest that preeclampsia induced periodontal disease rather than that periodontal disease induced preeclampsia. Also, the timing of periodontal treatment may not have been optimal. The clinical symptoms of preeclampsia are thought to be late manifestations of pathological processes in the first half of pregnancy[24;80]. Therefore, periodontal therapy at ±20 weeks of gestation may be too late in pregnancy to prevent preeclampsia. Moreover, translocation of microorganisms from the periodontal infection to the placental tissues may have occurred before therapy.

Although the present review does not undisputedly show that periodontal disease induces preeclampsia; there are various indications that periodontal disease may play a role in the pathogenesis of preeclampsia. Key pathogens associated with periodontal disease in adult subjects are the gram-negative microorganisms *Aggregatibacter*

actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Fusobacterium nucleatum and the gram-positive *Parvimonas micra*[82]. Some studies found a higher prevalence of *P. gingivalis*, *Eikenella corrodens* and *T. forsythia*[39;53] or *P. micra*[54] in subgingival plaque samples of preeclamptic women as compared with healthy pregnant controls. These bacteria produce a variety of pro-inflammatory factors, e.g. lipopolysaccharide (LPS)[83-86], which may further activate the normal inflammatory response during pregnancy, ultimately resulting in preeclampsia. Indeed, infusion of low doses of *Escherichia coli* LPS into pregnant rats leads to activation of the inflammatory response and subsequently to a preeclampsia-like syndrome[87]. Whether LPS of gram-negative oral pathogens is also capable of provoking preeclampsia-like symptoms is under investigation. Also, the bacteria themselves may also enter the circulation and affect the inflammatory response or tissues directly. Barak et al[37] found increased bacterial counts for all important periodontal pathogens in placentas of women with preeclampsia. The presence of periodontal pathogens in placental tissues of preeclamptic pregnancies may imply a role for these bacteria in the pathogenesis of preeclampsia.

Interestingly, prospective studies that performed periodontal examinations before 26 weeks of gestation failed to find an association[42;56]. In fact, although Boggess et al reported that periodontal disease at delivery or periodontal disease progression during pregnancy was associated with preeclampsia, periodontal disease at enrolment (gestational age <26 weeks) was not associated with an increased risk of developing preeclampsia[42]. This may suggest that periodontal disease does not induce preeclampsia. Although these studies were not designed to demonstrate that preeclampsia is a risk factor for the induction of periodontal disease, the possibility can not be ruled out that the preeclamptic state itself may have induced or aggravated periodontal problems, as was suggested above for the study of Kunnen et al[54]. This is in line with the two-hit model of Golub et al[88]. In this model, the first hit is initiated by detrimental microbial products arising from the periodontal biofilm. The second hit is the generalized inflammatory disease (in this case preeclampsia) with increased circulating levels of pro-inflammatory cytokines. Together these two hits could lead to an increase of local inflammatory mediators and effector molecules including matrix metalloproteinases, and finally to periodontal breakdown.

The present review shows that in order to further evaluate the relationship between periodontal disease and preeclampsia, there is great need for larger studies, with standardized protocols. It is especially important to use a universal standardized periodontal disease definition that includes the inflammatory burden and assesses the risk of systemic effects of periodontitis. Interestingly, such a model has recently been developed by Nesse et al: the PISA method (Periodontal Infected Surface Area)[66]. This model calculates the extensiveness of the infected periodontal surface in square

millimetres and can therefore quantify the total inflammatory burden. Also, RCTs should be performed that have preeclampsia as primary outcome. Moreover, it is recommended that future studies focus on dissecting the biological mechanisms that may link both conditions. Additional studies in terms of virulence properties of oral pathogens and subsequent host responses to these pathogens during pregnancy as well as pathophysiological studies investigating foetal exposure to periodontal microbiota and maternal immune responses are warranted. Since there is convincing evidence in non-pregnant individuals that systemic antibiotics improves periodontal health and has a long-term reducing effect on the bacterial infection[89], future research on the effects of periodontal therapy during pregnancy may include the prescription of antibiotics.

Clinical relevance

Scientific rationale for the study: Periodontal disease has been proposed to contribute to the pathogenesis of preeclampsia. Therefore, we reviewed the scientific evidence of the relationship between periodontal disease and preeclampsia.

Principal findings: There are indications of an association between periodontal disease and some forms of preeclampsia. This is most obvious in early-onset preeclampsia. However, it is unclear whether periodontal disease plays a causal role in preeclampsia, for this review not only shows that periodontal therapy performed at about 20 weeks of gestation did not reduce preeclamptic rates but also studies focusing on the periodontal condition at enrolment, i.e. before preeclampsia had occurred, failed to show an association. Therefore, periodontal disease at delivery may be the consequence of the preeclamptic state rather than vice versa. Inconsistent findings among the observational studies may be due to a large variety in periodontal disease definitions and timing of screening.

Practical implications:

Periodontal treatment during pregnancy did not seem to influence the risk of preeclampsia. Larger RCTs with standardized protocols and preeclampsia as the primary outcome, as well as pathophysiological studies are required to determine whether the observed relationship between preeclampsia and periodontal disease in some studies is causal or simply associative. So far, periodontal treatment during pregnancy, if needed, is not contraindicated.

Supporting information

Appendix 1, Supplemental Table S1: Dutch Cochrane Collaboration quality assessment checklist for case-control studies.

Appendix 1, Supplemental Table S2: Dutch Cochrane Collaboration quality assessment checklist for cohort-studies.

Appendix 1, Supplemental Table S3, Quality assessment checklist for cross-sectional studies, adapted from the Dutch Cochrane Collaboration quality checklist for cohort studies.

Appendix 1, Supplemental Table S4: Dutch Cochrane Collaboration quality assessment checklist for randomized-controlled trials.

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Appendix 1, Supplemental files S1-S4

Supplemental Table S1. Quality assessment of the case-control studies*

Study Quality criteria	Canakci et al (2004)	Oettinger-Barak et al (2005)	Contreras et al (2006)	Khader et al (2006)	Kunnen et al (2007)	Canakci et al (2007)	Siqueira et al (2008)	Lohsoon-thorn et al (2009)	Nabet et al (2010)	Shetty et al (2010)
Are the characteristics of the study group clearly defined?	+	–	+	+	+	+	+	+	+	+
Are the characteristics of the control group clearly defined?	+	–	+	+	+	+	+	+	+	+
Can selection bias sufficiently be excluded?	+	+	–	+	–	–	+	+	+	–
Are the exposure and method of assessment clearly defined (periodontal disease)?	+	+	+	+	+	+	+	+	+	–
Are the outcome and method of assessment clearly defined (preeclampsia)?	+	+	+	+	+	+/–	+	+	+/–	+
Is the exposure assessed blinded to disease status?	?	–	–	+	–	?	+	+	+/–	–
Are the most important confounders identified and are these taken into consideration with respect to the study design and analysis?	+	–	–	+	+	+	+	+	+	+
Reported odds ratios or risk ratios with 95% CI?	+	–	+	+	+	+	+	+	+	+

*Quality assessment according to the Dutch Cochrane Collaboration checklist for case-control studies.
Five or more plusses: methodologically acceptable.

Supplemental Table S2. Quality assessment of the cohort studies*

Study Quality criteria	Boggess et al (2003)	Meurman et al (2006)	Srinivas et al (2009)
Are the characteristics of the comparative study groups clearly defined?	+	–	+
Can selection bias sufficiently be excluded?	+	–	–
Are the exposure and method of assessment clearly defined (periodontal disease)?	+	+	+
Are the outcome and method of assessment clearly defined (preeclampsia)?	+	+	+
Is the outcome assessed blinded to the exposure status?	–	+	+
Is there a sufficient follow-up?	+	+	+
Can selective loss-to-follow-up sufficiently be excluded?	–	+	–
Are the most important confounders identified and are these taken into consideration with respect to the study design and analysis?	+	–	+
Reported odds ratios or risk ratios with 95% CI?	+	–	+

*Quality assessment according to the Dutch Cochrane Collaboration checklist for cohort studies.
Six or more plusses: methodologically acceptable.

Supplemental Table S3. Quality assessment of the cross-sectional study*

Study Quality criteria	Castaldi et al (2006)
Are the characteristics of the comparative study groups clearly defined?	–
Can selection bias sufficiently be excluded?	–
Are the exposure and method of assessment clearly defined (periodontal disease)?	+
Are the outcome and method of assessment clearly defined (preeclampsia)?	+
Is the outcome assessed blinded to the exposure status?	+
Are dropouts and reasons for dropout reported?	+
Are the most important confounders identified and are these taken into consideration with respect to the study design and analysis?	–
Reported odds ratios or risk ratios with 95% CI?	+

*Quality assessment adapted from the Dutch Cochrane Collaboration checklist for cohort studies.
Five or more plusses: methodologically acceptable.

Supplemental Table S4. Quality assessment of the randomized-controlled trials*

Study Quality criteria	Michalowicz et al (2006)	Herrera et al (2009)	Offenbacher et al (2009)	Newnham et al (2009)
Was the assignment of patients to the intervention randomized?	+	+	+	+
Were staff members who included patients aware of the randomization order?	?	+	?	+
Were patients blinded to the treatment?	–	–	–	–
Were dental therapists blinded to the treatment?	–	–	–	–
Were effect examiners blinded to the treatment?	+	–	+	+
Were the groups comparable at trial entry?	+	–	+	+
Is there a sufficient number of patients with a completed follow-up?	+	?	+	+
Are all the included patients analysed in the group of randomization?	+	+	+	+
Apart from the intervention, did both groups receive equal treatment?	+	+	+	+

*Quality assessment according to the Dutch Cochrane Collaboration checklist for randomized-controlled trials.
Six or more plusses: methodologically acceptable.

CHAPTER 3

Women with a recent history of early-onset preeclampsia have a worse periodontal condition

This chapter is an edited version of the manuscript:
Kunnen A, Blaauw J, van Doormaal JJ, van Pampus MG, van der Schans CP, Aarnoudse JG, van Winkelhoff AJ, Abbas F. Women with a recent history of early-onset pre-eclampsia have a worse periodontal condition. *J Clin Periodontol* 2007;34:202–207

Abstract

Objective: Preeclampsia is a complication of pregnancy characterised by systemic vascular dysfunction and pathological changes in placental arteries. Growing evidence of chronic infection as an aetiological factor in vascular diseases prompted us to study maternal periodontal disease in subjects with early-onset preeclampsia (<34 weeks).

Methods: A case-control study was carried out on 17 early-onset preeclamptic women and 35 controls with uncomplicated pregnancies in a period of 3-28 months postpartum. All were Caucasians. Full mouth periodontal examinations were performed to determine the periodontal condition. Subgingival plaque samples were analysed by anaerobic culture techniques for the presence of 7 bacterial periodontal pathogens. Potential confounders as age, smoking, educational level and body mass index were determined.

Results: Severe periodontal disease was found in 82% of the preeclamptic and in 37% of the control group (p=0.009). After adjusting for age, smoking and educational level, the odds ratio was 7.9 (95% CI: 1.9-32.8). The periodontopathic microorganism *M. micros* was more prevalent in the case group (p=0.040) while *C. rectus* was more prevalent in the control group (p=0.047).

Conclusion: These results indicate that Caucasian women with a recent history of early-onset preeclampsia have a worse periodontal condition, as compared with women with uncomplicated deliveries.

Introduction

There is growing evidence that periodontal disease is associated with adverse pregnancy outcome, e.g. preterm birth, low birthweight, miscarriage and preeclampsia as reviewed by Xiong and co-workers[1]. So far, most studies have been focused on the role of periodontal disease in preterm birth[2-6], including intervention studies suggesting that periodontal treatment reduces the risk of preterm birth[7;8]. Results of recent investigations have suggested that periodontal disease is more prevalent in preeclampsia[9-12]. Preeclampsia is a multisystemic maternal vascular disease with endothelial dysfunction, clinically manifest during the second half of pregnancy by hypertension, proteinuria and varying dysfunction of major organs as the liver, the kidneys and the brain. It is a major cause of both maternal and foetal mortality and morbidity[13-15]. Early-onset preeclampsia is a severe form of preeclampsia, occurring before 34 weeks of pregnancy and very often accompanied by restricted foetal growth[16] which is thought to be the result of insufficient placentation in the first half of pregnancy.

Until now, the mechanisms causing the maternal vascular abnormalities during the second half of pregnancy have not been fully clarified. The current opinion is that several mechanisms can result in the final common pathway of maternal endothelial dysfunction[17;18]. There is considerable evidence that one of these mechanisms is an exaggerated inflammatory response[19-21]. Chronic inflammation including periodontal disease, has been linked to atherosclerosis, another manifestation of vascular endothelial disease[22-24]. This prompted us to study maternal periodontal disease in subjects with early-onset preeclampsia (<34 weeks). Periodontal disease as well as preeclampsia are of multifactorial nature, including e.g. ethnicity and socioeconomic status. The reported studies on periodontitis and preeclampsia were carried out in ethnically heterogenous populations. The aim of the present study was to investigate the periodontal condition in a homogenous Caucasian population with a recent history of early-onset preeclampsia. To exclude direct possible hormonal influences caused by pregnancy, we compared the periodontal condition of women with a recent history of early-onset preeclampsia with a control group of women who recently had an uncomplicated pregnancy.

Materials and Methods

Participants

A case-control study was carried out in the northern part of the Netherlands to compare the periodontal status between women with previous early-onset preeclampsia (N=17) and women with an uncomplicated pregnancy (N=35) in a period of 3-28 months postpartum. Women who previously were referred to the Department of Obstetrics & Gynaecology, University Medical Center Groningen were invited for the present study.

Preeclampsia was defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy: the appearance of a diastolic blood pressure ≥90 mmHg measured at 2 occasions at least 4 hours apart in combination with proteinuria (≥300 mg/24 hours or 2+ dipstick) developing after a gestational age of 20 weeks in a previously normotensive woman. Early-onset preeclampsia was defined as preeclampsia occurring before 34 weeks of pregnancy. Healthy controls were recruited by advertisements in local news papers. Only women of Caucasian origin were included. All women have been under a pregnancy control program during their pregnancy by midwives or general physicians. Women with pre-existing hypertension (blood pressure before 20 weeks of gestation ≥140/90 mmHg or using anti-hypertensive medication), diabetes mellitus, renal disease, cardiovascular disease or any systemic illness, multiple pregnancy or post partum thyroiditis were excluded from the study. Patients who had received periodontal treatment in the past or patients on antibiotic medication during pregnancy or the post-partum period were also excluded, as well as women who were pregnant or breast feeding in the preceding three months before study-inclusion. All participants received information about the purpose of the study and provided informed consent before participation. After investigation, the subjects were informed about their periodontal status and advised on further treatment if indicated.

Data collection

Obstetric and medical history, educational level and health behaviour data of the preeclamptic women were obtained from the medical records of the Department of Obstetrics & Gynecology. Data of the control group were collected by questionnaire. This questionnaire was designed to gather data on medical history, previous pregnancy history (including gestational age at delivery, onset of delivery, birth weight and gender of the neonate), educational level, smoking habits and health behaviour data. Educational level was used as marker for socioeconomic status. Oral hygiene behaviour data were obtained by patient interview at the first visit.

Periodontal examinations

Participants underwent a full periodontal examination by one certified dental hygienist (AK). Clinical examinations were performed to determine the following variables: plaque index, bleeding on probing (BOP), pocket probing depth (PPD), clinical attachment level (CAL) and gingival recessions (GR). PPD and GR were measured in millimeters (to the nearest millimeter) with a Williams UNC-15 periodontal probe at six sites per tooth, excluding the third molars. PPD was defined as the distance from the gingival margin to the bottom of the pocket. GR was calculated from the distance from the cementoenamel junction (CEJ) to the gingival margin. CAL was measured in millimeters at two sites per tooth (buccal and lingual) and was defined as the distance from the

CEJ to the bottom of the pocket. CAL was calculated by distracting the distance of the gingival margin to the CEJ from the PPD, or in case of visible CEJ: PPD plus GR. BOP was expressed as the percentage of sites showing bleeding on probing. The periodontal condition was determined by the sum of all pockets with PPD ≥4 mm and BOP. The periodontal condition was further stratified in severity according to the criteria used by Boggess et al[9]. Periodontal health was defined as absence of pocket probing depths ≥4 mm. Mild periodontal disease was defined as 1 up to 15 tooth sites with ≥4 mm pocket depth and BOP. Severe periodontal disease was defined as ≥15 tooth sites with ≥4 mm pocket depth and BOP.

Microbiological procedures

In each subject gingival crevicular fluid samples were collected from the deepest periodontal pocket in each quadrant (for a total of 4 samples) after periodontal examination. Sterile paperpoints were inserted to the bottom of the periodontal pockets for 10 seconds and pooled in reduced transport fluid (Syed & Loesche[25]) and analysed by anaerobic culture techniques for the presence and levels of *Actinobacillus actinomycetemcomitans (*Aa), *Porphyromonas gingivalis, (*Pg*), Prevotella intermedia* (Pi), *Tannerella forsythensis* (Tf), *Micromonas micros* (Mm), *Fusobacterium nucleatum* (Fn) and *Campylobacter rectus* (Cr).

After vortexing for 30 secs, samples were tenfold serially diluted in RTF and 100 ul of appropriate dilutions were plated on non-selective 5% horse blood agar plates (Oxoid no. 2, Oxoid Ltd., Basingstoke, England) supplemented with haemin (5 mg/L) and menadione (1 mg/L) for enumuration of the total anaerobic bacterial count and to test for the presence and relative proportions of specific periodontal pathogens. Samples were also plated onto tryticase soy serum-bacitracin-vancomycin plates (TSBV, Slots[26]) for the isolation and enumeration of *Actinobacillus actinomycetemcomitans*. TSBV plates were incubated in air plus 5% CO_2 at 37°C for 5 days. Blood agar plates were incubated for 14 days at 37°C in 80% N_2, 10% CO_2 and 10% H_2.

Data processing and statistical analyses

All data were stored in and analysed using SPSS 12.0. Univariate association between periodontal disease and presence or absence of preeclampsia (nominal data) was assessed using chi-square test. Differences between the case and the control group were in case of normally distributed continuous data analysed by Student's *t*-test, and in case of nominal data by the chi-square test. Pearson correlation coefficients were calculated to assess the association between the periodontal condition and postpartum period. Multivariate logistic regression analysis was used to determine the association between periodontal disease and preeclampsia adjusted for potential confounders (smoking, body mass index (BMI), socioeconomic status and age). From the logistic regression

analysis odds ratios were calculated with 95% confidence interval (CI). $p<0.05$ was accepted as statistically significant.

Results

Of a total of 25 women with a history of early-onset preeclampsia who were asked to participate, 17 agreed to participate in the study. There was no evidence for selection bias in recruitment; in particular, participants and non-participants were of similar age and parity, and they had similar blood pressure before the index pregnancy. Of the 36 controls, one woman was excluded because of recent antibiotic medication.

Table 1 shows the maternal and obstetric characteristics of the participants. There were no differences between case- and control group, with exception of educational level and family history of cardiovascular disease.

Table 1. Maternal and obstetric characteristics of preeclamptic women and non-preeclamptic women

	Preeclampsia (N = 17)	Controls (N = 35)	*p* value
Age (year)	29.5 (5.1)	31.7 (4.2)	0.095
Body Mass Index (kg/m^2)	27.8 (6.6)	24.3 (3.9)	0.051
Family history of CVD	13 (77%)	11 (31%)	0.002
Primigravida	16 (94%)	35 (100%)	0.147
Gestational age at delivery (weeks)	30.7 (2.8)	39.9 (1.4)	<0.001
Birthweight child (gr.)	1133 (300)	3532 (417)	<0.001
Smoking during pregnancy	3 (18%)	3 (9%)	0.337
Educational level			0.020
Very low	0 (0%)	2 (5.7%)	
Low	1 (5.9%)	1 (2.9%)	
Moderate	11 (64.7%)	8 (22.9%)	
High	5 (29.4%)	24 (68.6%)	
Interval delivery to day of study (months)	14.0 (8.2)	15.7 (5.8)	0.434

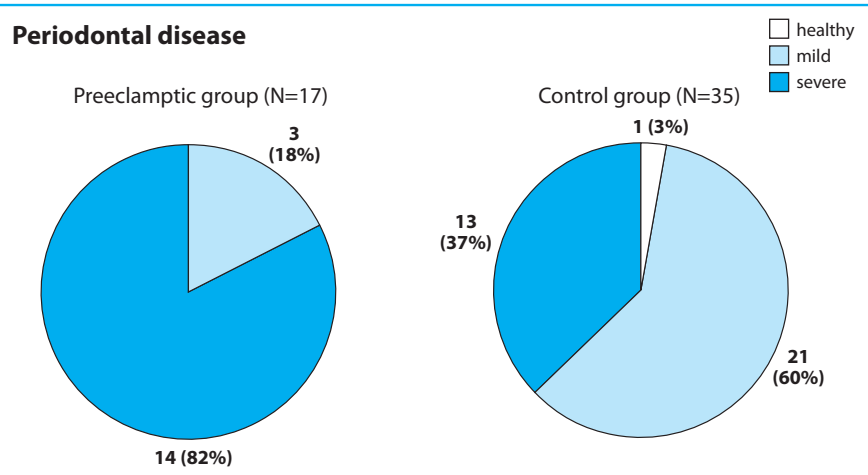
Values are expressed as means (SD) or number (%) unless otherwise stated.
CVD, cardiovascular disease.

The results of the periodontal examinations are presented in Table 2. The periodontal condition in the preeclamptic group was significantly worse compared with the controls. Severe periodontal disease was found in 14 of the 17 (82%) preeclamptic women and in 13 of the 35 (37%) women in the control group. Mild periodontal disease was found in three of the 17 (18%) preeclamptic women and in 21 of the 35 (60%) women in the control group (p=0.009). One person in the control group was periodontally healthy. After adjusting for age, BMI, smoking and educational level, the odds ratio was 7.9 (95% CI: 1.9-32.8) (Figure 1).

Table 2. Periodontal and clinical dental variables in preeclamptic and non-preeclamptic women

	Preeclampsia (N = 17) mean (SD)	Controls (N = 35) mean (SD)	*p* value
Mean PD (mm)	2.94 (0.37)	2.57 (0.31)	0.000
PD ≥ 4 mm	30.18 (18.48)	15.23 (13.12)	0.001
PD 4 mm	19.53 (8.57)	11.00 (7.77)	0.001
PD 5 mm	7.82 (7.33)	3.37 (5.59)	0.019
PD 6 mm	2.82 (4.49)	0.86 (2.58)	0.050
Mean CAL (mm)	1.38 (0.63)	1.10 (0.45)	0.073
% sites with bleeding	55.81 (20.41)	45.71 (20.45)	0.101
% sites with plaque	78.71 (15.18)	77.84 (17.11)	0.858
Tooth loss*	0.59 (0.80)	0.54 (1.12)	0.882

*Number of teeth lost excluding third molars.
CAL, clinical attachment level; PD, probing depth.



Odds ratio for severe periodontal disease = 7.9 (95% CI: 1.9-32.8).

Figure 1. Association between periodontal disease and presence or absence of preeclampsia (*p*=0.009).

To assess bias caused by possible third molar defects or eruption problems, we also determined the periodontal condition with exclusion of the distal sides of the second molars. Severe periodontal disease was then found in 13 of the 17 (76%) preeclamptic women and in 12 of the 35 (34%) women in the control group while mild periodontal disease was found in four of the 17 (24%) preeclamptic women and in 22 of the 35

(63%) women in the control group (p=0.016). The odds ratio then became 6.2 (95% CI: 1.7-23.3). No association was found between postpartum period and the periodontal condition.

There was no significant difference in the presence of periodontopathic micro-organisms between cases and controls, with exception of *M. micros*, which was detected more frequently in the case group (p=0.040) and *C. rectus*, which occurred more often in the control group (p=0.047) (Table 3). Two of the microbiological samples were lost during transport.

Table 3. Prevalence of periodontopathic micro-organisms in preeclamptic and non-preeclamptic women

Micro-organism	Preeclampsia (N = 17) n (%)	Controls (N = 35) n (%)	*p* value
Red complex			
P. gingivalis	1 (6.7)	2 (5.7)	0.777
T. forsythensis	12 (80)	22 (62.9)	0.283
Orange complex			
P. intermedia	3 (20)	7 (20)	0.709
F. nucleatum	15 (100)	34 (97.1)	0.158
M. micros	15 (100)	25 (71.4)	0.040
C. rectus	1 (6.7)	5 (14.3)	0.047
Green complex			
A. actinomycetemcomitans	2 (13.3)	1 (2.9)	0.698

Discussion

This study shows that Caucasian, western European women with a recent history of early-onset preeclampsia have a worse periodontal condition compared with healthy controls. These findings are consistent with previous findings that periodontal disease is associated with preeclampsia[9-12]. Caution is needed in interpreting these results due to the limited number of cases. Until now, no conflicting results have been reported in past studies on an association between preeclampsia and periodontal disease. This is in contrast with studies on an association between periodontal disease and preterm birth, where findings throughout the years have not been consistent. Several potential biases have been noted among past studies, with the most important being the great variation in periodontal disease definitions[1]. To overcome this problem, we tried to obtain consistency by stratifying periodontal disease according to the definitions used by Boggess et al[9]. Classifying periodontal disease on PPD ≥4 mm, however, might not

be discriminating enough with respect to the severity of periodontal disease and might lead to overestimation of the disease. In this respect it is interesting to mention a recent study in which different periodontal measurements and/or definitions are compared in relation to earlier observed associations between periodontal disease and risk of myocardial infarction[27]. Results of this case-control study show that the observed associations remained consistent across different periodontal measurements/ definitions. The association was strongest when PPD was used to measure periodontal disease.

Furthermore, our study was carried out postpartum, while the studies in the USA[9], Turkey[10], Israel[12] and Colombia[11] were carried out during pregnancy. This and the wide periodontal examination period (3-28 months postpartum) in our study merit discussion. The periodontal status after delivery might have changed over this period of time. None of the women in the case or control group reported specific periodontal treatment. With no intervention it is unlikely to expect spontaneous improvement in periodontal condition or bacterial load[28]. Since we excluded women who were pregnant of breast-feeding within three months prior to study-inclusion, hormonal influences on the periodontal condition are no longer to be expected. No correlation was found among the postpartum period and the periodontal condition. This suggests that there has been no systematic improvement or worsening of the periodontal condition postpartum. However, periodontal disease status might have worsened in the individual patient throughout the postpartum period.

The background of the connection between periodontal disease and preeclampsia is far from clear. There are several possibilities. Firstly, preeclampsia and periodontal disease have risk factors in common. Although we accounted for these common risk factors by exclusion of patients of black race and patients with diabetes mellitus, and by correction for low socioeconomic status in the statistical analyses, we can not rule out the possibility of an unknown factor that predisposes to both preeclampsia and periodontal disease. This unknown factor might be a genetic one involved in the process of atherosclerosis, since a family history of cardiovascular disease was more prevalent in the preeclamptic group and a history of preeclampsia as well as periodontal disease increase the risk of atherosclerotic manifestations[29-33]. Remarkably, the prevalence of smoking is high and nearly the same in the preeclamptic group as in the control group. Smoking, however, is a risk factor for periodontal disease[34], poor foetal growth[35] and preterm birth[36], but seems to reduce the risk of developing preeclampsia[37]. This unexpected high rate of smoking in the preeclamptic group could have contributed to the high prevalence of severe periodontal disease in this group.

Secondly, it is possible that preeclampsia leads to an aggravation of pre-existing periodontal problems or even co-induces periodontal disease[38]. Alterations in the maternal immune system have been reported in preeclampsia. Recent studies demon-

strate altered Th1-cytokine and CD4 cell expression in preeclampsia[39-41]. Th1-cytokines and CD4 cells play an important role in controlling ongoing infections and the host's tissue destruction seen in periodontal disease progression[42].

Thirdly, pre-existing (severe) periodontal disease might play a role in the initiation and progression of early-onset preeclampsia, since the presence of a chronic infection during pregnancy is thought to be a risk factor for preeclampsia[43;44]. It has been hypothesised that periodontal disease generates an inflammatory reaction leading to elevated systemic levels of cytokines, such as tumour necrosis factor-alpha (TNF-α), prostaglandin E_2 (PGE_2), interleukin-1β and interleukin-8[45]. This host response to a long-term exposure of periodontal pathogens may provoke systemic maternal and placental pro-inflammatory endothelial activation and dysfunction, which represent a significant risk factor for diseases of vascular origin, such as preeclampsia[17;18;20;46].

The prevalence of the periodontal pathogen *M. micros* was significantly higher in the preeclamptic group, while *C. rectus* was found more often in the control group. *M. micros* is a known bacterial marker for destructive periodontal disease in adult subjects[47]. The higher prevalence of *M. micros* in the preeclamptic group may be explained by the worse periodontal status in this group. *M. micros* and *C. rectus* are both members of the 'orange' complex, which is one of the two complexes thought to consist the major etiologic agents associated with periodontal disease[48]. Recently, Skuldbøl et al[49] found *M. micros* significantly more often in the subgingival plaque of women with pre-term birth in comparison to women with term birth[49]. Buduneli et al[50] and Madianos et al[51] also evaluated the microbiological differences in subgingival plaque between preterm and full-term mothers. They found no significant differences in the prevalence of the individual periodontopathic micro-organisms between cases and controls, although Madianos found elevated levels of foetal IgM to *C. rectus* among the premature infants[51]. However, when multiple micro-organisms of the subgingival plaque were evaluated together, regression analysis indicated that the presence of both *M. micros* and *C. rectus* might lead to an increased risk on preterm birth[50]. The authors suggest that this might be explained by complex actions of different micro-organisms rather than the presence of individual species. The results of our present study are not congruent with the results of these articles and do not support this hypothesis. The role of *M. micros* in the development of preeclampsia remains therefore unclear.

In case of a causal link between periodontal disease and preeclampsia, periodontal treatment is to be expected to reduce the risk of preeclampsia, like periodontal treatment reduces the risk of preterm birth[7;8]. This hypothesis needs to be tested. Since the host response to periodontal pathogens might play a key role in the development of preeclampsia, further research on the role of the inflammatory reaction as plausible mechanism is required.

In summary, women with a recent history of early-onset preeclampsia have a worse periodontal condition. Thereby, patients and physicians should be aware of the possible relationship between periodontal disease and adverse pregnancy outcomes, such as preeclampsia.

Acknowledgements

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Clinical relevance

Scientific rationale for the study: Chronic inflammation, including periodontal disease, is thought to play a role in the development of preeclampsia, a serious pregnancy complication. We investigated the periodontal status in women with a recent history of severe early-onset preeclampsia and compared them to women with an uncomplicated pregnancy.

Principal findings: Women with a recent history of early-onset preeclampsia showed a worse periodontal condition, after adjusting for smoking, BMI, age and socioeconomic status.

Practical implications: Screening for periodontal disease and treatment of affected women might effectively prevent adverse pregnancy outcomes, such as preeclampsia, if causality of periodontal disease is evidenced.

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CHAPTER 4

The effect of Porphyromonas gingivalis lipopolysaccharide on pregnancy in the rat

This chapter is an edited version of the manuscript:
Kunnen A, van Pampus MG, Aarnoudse JG, van der Schans CP, Abbas F, Faas MM. The effect of *Porphyromonas gingivalis* lipopolysaccharide on pregnancy in the rat. *Oral Diseases* (accepted for publication, 2013)

Abstract

Objective: Periodontitis, mostly associated with *Porphyromonas gingivalis*, has frequently been related to adverse pregnancy outcomes. We therefore investigated whether lipopolysaccharides of *P. gingivalis* (Pg-LPS) induced pregnancy complications in the rat.

Methods: Experiment 1: pregnant rats (day 14) received increasing Pg-LPS doses (0.0-50.0 µg/kg bw; n=2/3 p/dose). Maternal intra-aortic blood pressure, urinary albumin excretion, placental and foetal weight and foetal resorptions were documented. Experiment 2, 10.0 µg/kg bw (which induced the highest blood pressure together with decreased foetal weight in experiment 1) or saline was infused in pregnant and non-pregnant rats (n=7/9 p/group). Parameters of experiment 1 and numbers of peripheral leukocytes as well as signs of inflammation in the kidney and placenta were evaluated.

Results: Pg-LPS infusion in pregnant rats increased maternal systolic blood pressure, reduced placental weight (dose dependently) and decreased foetal weight and induced foetal resorptions. It, however, did not induce proteinuria or a generalised inflammatory response. No effects of Pg-LPS were seen in non-pregnant rats.

Conclusion: Pg-LPS increased maternal blood pressure, induced placental and foetal growth restriction and increased foetal resorptions, without inducing proteinuria and inflammation. Pg-LPS may therefore play a role in pregnancy complications induced by periodontitis.

Introduction

Porphyromonas gingivalis has been identified as a major aetiological factor in the pathogenesis of periodontitis[1]. Periodontitis is not only a local infection, but periodontal pathogens, especially *P. gingivalis*[2], can also be found at distant sites[3] or induce changes in systemic inflammatory parameters[4], for instance increased plasma C-reactive protein (CRP) levels[5] as well as activated inflammatory cells and endothelial cells[6]. Indeed, periodontitis has been associated with various systemic diseases, such as atherosclerosis, myocardial infarction and cardiovascular diseases (CVD)[7]. The systemic nature of periodontitis can also be seen from the putative relationship between maternal periodontitis and poor obstetric outcome[8-10].

A large number of observational studies showed a relationship between pregnancy outcome and periodontal disease. Periodontal disease during pregnancy has been associated with preterm birth, which is defined as a delivery before 37 weeks of gestation[10], low birth weight, which is defined as infants with birth weights of <2500 g[9], miscarriage or stillbirths[10] and preeclampsia[8]. Preeclampsia is one of the most important pregnancy complications in the western world and is characterised by maternal hypertension and proteinuria clinically manifest during the second half of pregnancy[11]. It has been suggested that the chronic general low-grade inflammation which is seen in patients with periodontitis may play a role in the pathogenesis of these pregnancy-related pathologies[12]. Although the exact relationship between periodontitis and pregnancy complications and mechanisms needs to be established, it seems likely that *P. gingivalis* is involved in inducing adverse pregnancy outcomes, since *P. gingivalis* has the capacity to disseminate from the oral cavity to the foetoplacental unit[13-17]. In animal experiments, *P. gingivalis* has been found in placental tissues after intravenous infection and appeared to induce lesions similar to human chorioamnionitis and placentitis[18].

Moreover, *P. gingivalis* in mice and rats was associated with foetal and placental growth restriction, increased foetal resorptions and foetal death[19;20]. *P. gingivalis* bacteria contain factors that are pro-inflammatory and can also potentially affect placental tissue. One of the most important factors is lipopolysaccharide (LPS)[21]. Indeed, intravenous infusion with LPS of *P. gingivalis* (Pg-LPS) in pregnant animals induced foetal growth restriction and foetal death[22]. Interestingly, pregnant individuals are extremely sensitive to LPS of *Escherichia coli* (Ec-LPS) and in recent animal studies, this LPS has been shown to induce not only placental effects[23], but also peripheral effects such as hypertension, proteinuria and generalised inflammation[24;25].

The aim of the present study therefore was to investigate whether Pg-LPS in pregnant rats also induced systemic complications, such as hypertension, proteinuria and generalised inflammation next to affecting placental and foetal growth.

Materials and methods

Handling of the rats

Animal experiments were conducted under protocols approved by the Animal Ethical Committee of the University of Groningen, the Netherlands. Female Wistar outbred rats (175-200 g) were obtained from Harlan Inc., Zeist, The Netherlands. Rats were maintained in a temperature- and light-controlled room (22°C, 12:12H light:dark cycle), with free access to standard laboratory chow and water. Animals were allowed seven days to acclimatise to the laboratory environment and were housed together until selection for experiments. After acclimatisation, vaginal smears were taken to follow oestrus cyclicity. On pro-oestrus, rats were housed overnight with two fertile Wistar outbred males. A positive vaginal smear for spermatozoa the next day was defined as day 0 of pregnancy. On day 0 of pregnancy and on diestrus-2 in non-pregnant control rats, a permanent cannula was surgically inserted into the right jugular vein under isoflurane/oxygen anaesthesia, according to standard methods[26]. This cannula allows stress-free infusion of either endotoxin or saline, and stress-free blood sampling. Rats were then housed individually and allowed to recover from surgery for 14 days. On day 14 of pregnancy (14 days after surgery in non-pregnant rats), rats were infused with either with 2.0 ml endotoxin solution (concentrations ranging from 1.0 to 50.0 µg Pg-LPS/kg bw in saline) or 2 ml saline alone during 1 hour via the permanent jugular vein cannula. Ultrapure lipopolysaccharide of *Porphyromonas gingivalis* (tlrl-pglps, strain ATCC 33277; InvivoGen, San Diego, USA) was used in this study.

Experimental design

Experiment 1: In this experiment, pregnant rats were infused with Pg-LPS at increasing doses from 0.0 to 50.0 µg Pg-LPS/kg bw (n=2/3 per dose) in order to establish a putative dose which would induce systemic, placental and foetal signs. Twenty four hour urinary albumin excretion was measured one day before infusion and at one and five days after infusion. On day 21, which is the end of pregnancy, and seven days after Pg-LPS infusion (in none of the rats parturition had started), rats were anaesthetised and aortic blood pressure was measured after which the rats were sacrificed by aortic puncture. Living foetuses and their placentas were collected and weighed. Total number of foetuses and the number of foetal resorptions were also counted. The percentage of foetal resorptions was calculated.

Experiment 2: From experiment 1 it appeared that Pg-LPS induced hypertension, decreased placental weight and increased the number of foetal resorptions. To further evaluate the effect of Pg-LPS on pregnant rats, in the second experiment, rats were infused with 10.0 µg/kg bw Pg-LPS, the dose that appeared to induce the highest blood pressure and also affected placental and foetal weight. The pregnant Pg-LPS (10.0 µg/kg bw) group (n=9), as well as the pregnant control group were expanded, which was

infused with 2.0 ml saline alone (n=9). Blood pressure, albumin excretion, foetal and placental weight were measured as in experiment 1. In addition, in order to investigate possible effects of Pg-LPS on the inflammatory response, we evaluated the number of peripheral white blood cells (WBC) as well as differential WBC counts and also signs of inflammation in the placenta and the kidney were evaluated. We choose to study these organs, since these organs are usually affected in pregnancy complications, such as preeclampsia. To explore whether the response to Pg-LPS was specific for pregnancy, also two groups of non-pregnant rats were included which were infused with either Pg-LPS (10.0 µg/kg bw in 2 ml saline) (n=7), or with 2.0 ml saline alone (n=7) using an identical experimental setup.

Blood pressure

For measuring intra-aortic blood pressure, at the end of pregnancy, a midline ventral incision was made under standard isoflurane/oxygen anaesthesia and the abdominal aorta was exposed. Immediately after exposure of the aorta, a needle connected to the probe of a pressure recorder (Lifescope 9, Bedside monitor MU-832 RK, Nihon Kohden Corporation, Tokyo, Japan) was inserted into the abdominal aorta after which the systolic and diastolic blood pressure were recorded. Rats were bled immediately afterwards by aortic puncture.

Albumin excretion

One day before the start of the infusion, as well as one day and five days after the infusion, rats were placed in metabolic cages (from 10.00 AM until 10.00 AM the next day) to collect a 24 hour urine sample. Urinary volume was measured and urinary albumin levels were assayed by the rocket-electrophoresis technique using rabbit-anti-rat albumin antiserum (Nordic Immunology, Tilburg, The Netherlands) according to the method of Laurell[27].

Tissue preparations

Immediately after sacrifice, the pregnant uterus was transected longitudinally to determine the number of living foetuses and resorptions. Each living individual foetus was weighed. In order to investigate if leukocyte infiltration occurred not only in placental tissue, but also in maternal tissue, from each dam, according to standard procedures, three representative placentas (from live foetuses) with associated mesometrial triangle were harvested, cut sagittally into two halves and snap frozen in isopentane (-80°C) and stored at -80°C for later analysis. Remaining placentas from the living foetuses (without mesometrial triangle) were weighed individually. Also from each dam, the left kidney was removed and collected. Kidney fragments were snap frozen immediately and stored at -80°C for later analysis.

White blood cell counts

Blood samples (0.1 ml in 12% ethylenediaminetetraactetate (EDTA)) were drawn from the permanent jugular vein cannula on day 14 (just prior to the start of the infusion), on day 15, 16, 19 and on day 21 between 09.00 and 11.00 AM. Twenty microlitre of blood was used to determine total WBC count on a microcell counter (model Sysmex pocH-100i Haematology Analyser, Sysmex Corp., Kobe, Japan). In order to determine differential WBC counts from each blood sample, a smear was made on a microscope slide and stained using the Giemsa method (1:10 dilution; Merck) for 20 minutes[28]. After evaluation of 200 cells per smear, relative and absolute numbers of granulocytes, lymphocytes and monocytes were calculated according to standard methods.

Immunohistochemistry

Cryostat placenta sections (along with the mesometrial triangle) and kidney sections (5 µm) were cut using a Leica CM1900 cryostat (Leica Microsystems, Wetzlar, Germany). The sections were collected on silane coated glass slides (Starfrost, Waldemar Knittel, Braunschweig, Germany) and allowed to air dry at room temperature and then stored at -20°C for later analysis.

Staining for granulocytes and monocytes/macrophages

Glomerular and placental sections were stained for granulocytes and monocytes/macrophages using primary monoclonal antibodies against rat granulocytes (His48; Becton Dickinson, NJ, USA) and primary monoclonal antibodies against rat monocytes/macrophages (ED1/CD68; AbD Serotec, Düsseldorf, Germany, dilution 1:100) according to standard protocols. Briefly, after drying in air, sections were fixed in pre-cooled acetone (4°C) for 10 minutes. Subsequently, sections were air dried and then incubated with the first antibody for one hour in the dark at room temperature. After washing in phosphate-buffered saline (PBS), endogenous peroxidase activity was blocked by incubation of the slides for 20 minutes in methanol supplemented with 3% hydrogen peroxide (H_2O_2). Thereafter, sections were incubated with normal rabbit serum (DAKO, Glostrup, Denmark, dilution 1:10) for 30 minutes. Then without rinsing, sections were incubated for 30 minutes with a Peroxydase-conjugated secondary antibody rabbit-anti-mouse (DAKO, Glostrup, Denmark, dilution 1:50). After washing in PBS, the reaction product was visualised using 3-amino-9-ethyl-carbazole (AEC; Sigma-Aldrich, St. Louis, MO, USA), counterstained with hematoxylin (1 min.) and embedded in Kaiser's glycerin-gelatin. Control sections not incubated with the primary antibody were consistently negative.

Evaluation of kidney sections

Kidney sections of each individual rat were quantitatively scored by light microscopic

examination. Sections stained for the presence of granulocytes and monocytes/macrophages were quantified by counting the total number of positive cells in 100 glomeruli per section, as described previously[29]. Results were expressed as the median of number of positive cells per glomerulus.

Evaluation of placental sections

Granulocyte and monocyte/macrophage infiltration were evaluated in the placentas and their associated mesometrial triangle by light microscopic examination.

Statistics

Statistical analysis was performed using SPSS 20 software for Windows. Results are expressed as individual values or illustrated in box and whisker plots. In experiment 1, Pearson correlation coefficients were calculated to evaluate the relationship between dose of Pg-LPS and blood pressure, placental weight or foetal weight. R^2 and the slope were calculated and it was tested whether the slope was statistically significantly different from zero. A slope which was different from zero indicated a dose response effect. If no dose response effect was observed, an effect of Pg-LPS was evaluated by grouping the data of all Pg-LPS infused rats together. These grouped data of the Pg-LPS infused rats were then compared with the saline infused rats using the Mann-Whitney *U* test. Before starting experiment 2, a power analysis was performed using maternal blood pressure as our main endpoint. The power analysis was based on the blood pressure data of experiment 1, with a mean systolic blood pressure of control pregnant rats of 86.3 mmHg and a variance of 12%. Based on the systolic blood pressure of Pg-LPS infused rats (Figure 1a), a 25% increase in mean systolic blood was expected. Using a power of 0.9 and a probability level of 0.05, power analysis showed that a minimum of 6 rats per group should be included. In experiment 2, differences between groups were tested by using the Mann-Whitney *U* test. Nominal pregnancy outcome data (number of resorptions and/or number of foetuses with growth restriction) were tested by using the Fisher's exact test. Post-infusion albumin excretion, total WBC counts and differential WBC counts were tested versus pre-infusion values using the Wilcoxon Signed Rank test. In the case of multiple comparisons, Bonferroni correction was used. $P<0.05$ was accepted as statistically significant.

Results

Experiment 1

Systolic and diastolic blood pressure and urinary albumin excretion

Figures 1 (a, b) show the aortic systolic and diastolic blood pressure of pregnant rats at gestational day 21, which is seven days after infusion with increasing doses of Pg-LPS (0.0-50.0 µg/kg bw Pg-LPS). No dose-response relationship between dose Pg-LPS and

systolic blood pressure (R^2=0.039, p=0.29) or diastolic blood pressure (R^2=0.013, p=0.56) was observed. However, when all Pg-LPS infused rats were grouped together, both diastolic and systolic blood pressure of Pg-LPS infused rats were significantly higher as compared with saline infused rats (p<0.05, Mann-Whitney U test). Urinary albumin excretion after infusion with increasing doses of Pg-LPS in pregnant rats is presented in Figure 1c. Pregnant rats did not exhibit increased albumin excretion after infusion with saline or Pg-LPS at any dose tested at one day after infusion (gestational day 15) or five days after infusion (gestational day 19) as compared with pre-infusion values (gestational day 13).

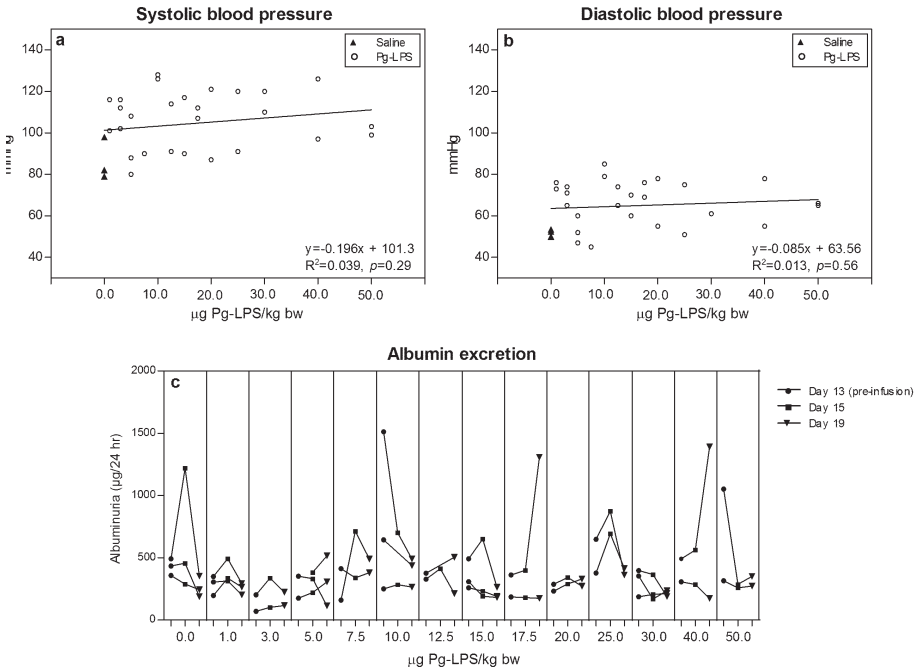

Figure 1. (**a**) Individual intra-aortic systolic blood pressure (**b**) and diastolic blood pressure of pregnant rats under standard isoflurane/oxygen anaesthesia on gestational day 21, seven days after infusion with increasing doses of Pg-LPS (0-50.0 µg/kg bw, n=2/3 p. dose). Closed triangles: saline control rats; open dots: Pg-LPS infused rats (R^2=Pearson correlation coefficients; p<0.05 indicates that slopes are significantly different from zero); (**c**) individual 24 hour urinary albumin excretion of pregnant rats after infusion with increasing doses of Pg-LPS (0-50.0 µg/kg bw, n=2/3 p. dose) at gestational day 13 (pre-infusion values: closed dots), gestational day 15 (one day after infusion: closed squares) and gestational day 19 (five days after infusion: closed triangles).

Placental and foetal weight

After infusion with 0.0-50.0 µg/kg bw Pg-LPS, a decreasing mean placental weight was observed with increasing Pg-LPS doses (R^2=0.218, p=0.005) (Figure 2a). There was no

significant dose response effect of increasing Pg-LPS doses and mean foetal weight (R^2=0.015, p=0.49) (Figure 2b). No significant differences in foetal weight were observed between saline infused rats and all Pg-LPS infused rats grouped together.

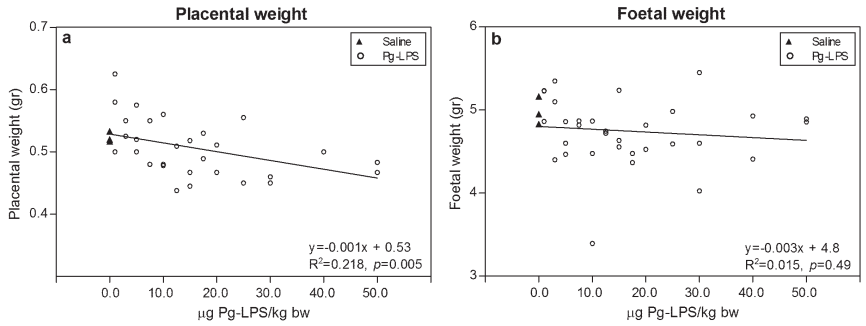

Figure 2. Placental (**a**) and foetal (**b**) weight after sacrifice at gestational day 21, seven days after infusion with increasing doses of Pg-LPS (0-50.0 µg/kg bw, n=2/3 p. dose). Each triangle/dot represents the mean placental or foetal weight per individual mother. Closed triangles: saline infused control rats; open dots: Pg-LPS infused rats (R^2=Pearson correlation coefficients; p<0.05 indicates that slopes are significantly different from zero).

Foetal resorption

Table 1 shows the total number of foetuses as well as percentages of foetuses resorpted (disintegrated and assimilated dead foetuses in the uterus) in the animals infused with the different doses of Pg-LPS. As can be seen from this table, foetal resorption was exclusively found after infusion with doses of Pg-LPS at or above 7.5 µg/kg bw.

Table 1. Foetal resorptions at gestational day 21 following infusion with saline or increasing doses of Pg-LPS

Pg-LPS (µg/kg bw)	No. of mothers	R/T	Resorptions (%)
0.0	3	0/32	0
1.0	3	0/31	0
3.0	3	0/34	0
5.0	3	0/39	0
7.5	2	2/25	8.0
10.0	3	2/35	5.7
12.5	2	1/27	3.7
15.0	3	0/39	0
17.5	2	1/26	3.8
20.0	2	1/21	4.8
25.0	2	0/25	0
30.0	3	8/29	27.6
40.0	2	0/24	0
50.0	2	1/22	4.5

R/T: number of resorptions/total number of resorbed and living foetuses

Experiment 2

Systolic and diastolic blood pressure and urinary albumin excretion

As infusion of 10 µg/kg bw Pg-LPS appeared to induce increased blood pressure together with decreased foetal weight, we chose this dose of LPS to further evaluate the effect of this LPS on pregnant animals. Figures 3 (a, b) show the aortic systolic and diastolic blood pressure of non-pregnant and pregnant rats after infusion with saline or 10.0 µg/kg bw Pg-LPS. It can be seen from these figures that infusion with 10.0 µg/kg bw Pg-LPS significantly increased systolic blood pressure in pregnant rats as compared with the saline infused pregnant controls ($p<0.05$, Mann-Whitney *U* test). This increase in systolic blood pressure after infusion with 10.0 µg/kg bw Pg-LPS did not occur in non-pregnant rats.

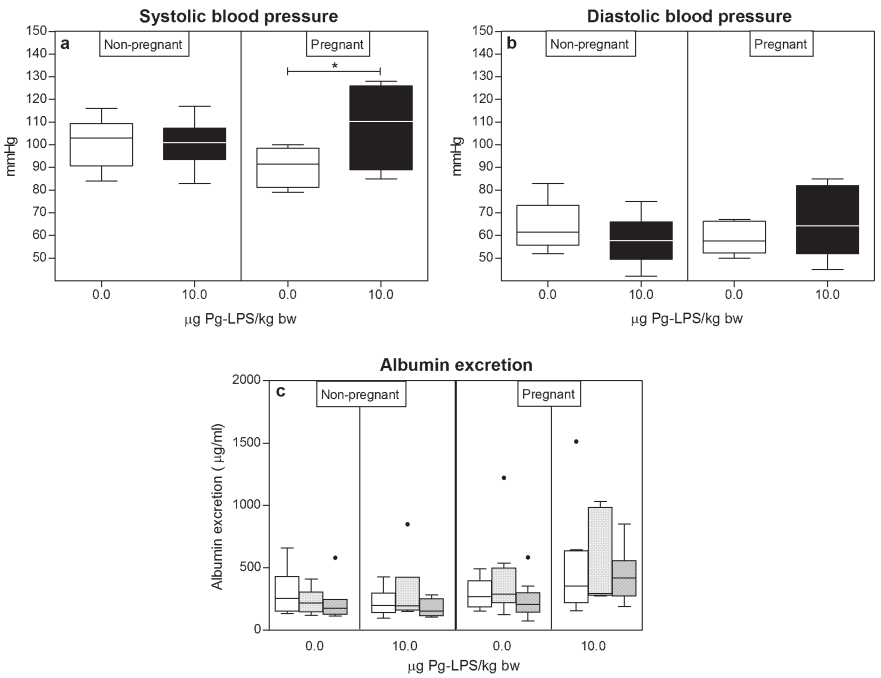

Figure 3. (**a**) Median and 25th and 75th percentiles of intra-aortic systolic and (**b**) diastolic blood pressure under standard isoflurane/oxygen anaesthesia of non-pregnant and pregnant rats after infusion with saline or 10.0 µg Pg-LPS/kg bw, seven days after infusion (gestational day 21 of pregnant rats). Open bars: saline infused control rats; black bars: Pg-LPS infused rats; (**c**) median and 25th and 75th percentiles of 24 hour albumin excretion of non-pregnant and pregnant rats after infusion with saline or 10.0 µg Pg-LPS/kg bw. Open bars: day 13 (pre-infusion values); dotted bars: day 15 (one day after infusion); striped bars: day 19 (five days after infusion). Error bars demonstrate 1.5 interquartile range and outlier values are represented as individual black dots. * Significantly increased vs saline infusion (Mann-Whitney *U* test; $p<0.05$).

No significant differences were observed in diastolic blood pressure in non-pregnant or pregnant rats infused with 10.0 µg/kg bw Pg-LPS vs saline infusion. Figure 3c shows 24 hour albumin excretion of non-pregnant and pregnant rats after infusion with saline or 10.0 µg/kg bw Pg-LPS. There was no difference in urinary albumin excretion after infusion with saline or 10.0 µg/kg bw Pg-LPS in non-pregnant or pregnant rats one day after infusion (gestational day 15 in pregnant rats) or five days after infusion (gestational day 19) as compared with pre-infusion values.

Placental and foetal weight

Infusion with 10.0 µg/kg bw Pg-LPS into day 14 pregnant rats significantly decreased placental weight as compared with the saline infused controls (median 0.5 [interquartile range (IQR) 0.1] gr saline vs median 0.4 [IQR 0.1] gr Pg-LPS, $p<0.05$, Mann-Whitney *U* test) (Figure 4a). Although there were no differences in median foetal weight between the Pg-LPS infused rats and the saline controls, there was a substantial amount of smaller than normal foetuses in the litter of the Pg-LPS infused group, as can be seen from Figure 4b. We therefore analysed the numbers of foetuses that were more than two standard deviations (2 SD) from the normal mean (4.75 ± 0.50 g; i.e. less than 3.75 g) and defined them as foetal growth restricted. Of 102 foetuses in the Pg-LPS infused group, 11 foetuses were growth restricted, while in the control group, only two of the 103 foetuses showed growth restriction. The number of growth restricted foetuses were significantly increased in LPS infused pregnant rats as compared to saline infused pregnant rats ($p<0.05$, Fisher's exact test).

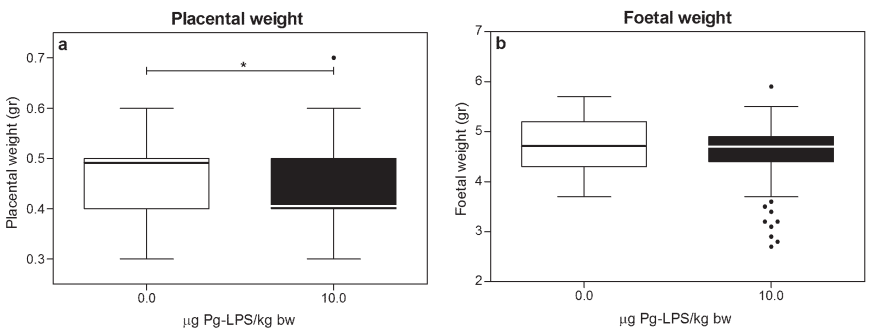

Figure 4. Median and 25th and 75th percentiles of placental (**a**) and foetal (**b**) weight after sacrifice at gestational day 21, seven days after infusion with saline or 10.0 µg Pg-LPS/kg bw. Error bars demonstrate 1.5 interquartile range and outlier values are represented as black dots. Open bars: saline infused control rats; black bars: Pg-LPS infused rats. * Significantly decreased vs saline infusion (Mann-Whitney *U* test; $p<0.05$).

Foetal resorption

In saline control rats, no foetal resorptions were observed. However, after infusion with 10.0 µg/kg bw Pg-LPS foetal resorptions were observed: of the total of 109 foetuses in 9 mothers, 7 foetuses were resorbed ($p<0.05$, Fisher's exact test).

Inflammatory parameters

Total WBC and granulocyte, monocyte and lymphocyte counts

Figure 5a shows total numbers of WBC as well as the differential counts for granulocytes, monocytes and lymphocytes at gestational day 14, i.e. before the infusion with 10.0 µg/kg bw Pg-LPS or saline. Total WBC counts before infusion were not significantly different in day 14 pregnant rats as compared with non-pregnant rats. In pregnant rats, total numbers of lymphocytes were decreased while total numbers of granulocytes were increased as compared with non-pregnant controls ($p<0.05$, Mann-Whitney *U* test).

Figures 5 (b, c) show the pre-infusion and post-infusion total WBC counts in non-pregnant rats and pregnant rats after infusion with 10.0 µg/kg bw Pg-LPS or saline. In both non-pregnant and pregnant rats, no differences between pre-infusion (gestational day 14 in pregnant rats) WBC counts and WBC counts on days 15, 16, 19 and 21 (i.e. one, two, five and seven days post-infusion, respectively) were observed in either the Pg-LPS treated rats or saline treated rats. There were no effects of infusion of either saline or 10.0 µg/kg bw Pg-LPS on the percentages of monocytes, lymphocytes and granulocytes in both non-pregnant and pregnant rats (results not shown).

Glomerular granulocyte and monocyte/macrophage infiltration

Figures 5 (d, e) show the number of glomerular granulocytes and glomerular monocytes/macrophages in non-pregnant and pregnant rats after infusion with saline or with 10.0 µg/kg bw Pg-LPS. We observed no effect of Pg-LPS on glomerular granulocyte or monocyte/macrophage number.

Placental granulocyte and monocyte/macrophage infiltration

In control placental tissues, only a few scattered granulocytes and monocytes/macrophages were present in the labyrinth and in the spongiotrophoblast layer. In the decidua basalis as well as in the mesometrial triangle, in particular in the central part of the mesometrial triangle and in close proximity of the spiral arteries, ED1 positive monocytes/macrophages were observed (Figure 6a). Granulocytes were found scattered in the mesometrial triangle (Figure 6b). However, when comparing the placental tissues of the 10.0 µg/kg bw Pg-LPS infused rats with the saline infused controls, no differences between the groups were observed in numbers of granulocytes and monocytes/macrophages (results not shown).

a. Median (interquartile range) of total and differential white blood cell (WBC) counts in non-pregnant and pregnant rats (gestational day 14), prior to infusion with saline or 10.0 µg Pg-LPS/kg bw

WBC	Non-pregnant rats		Pregnant rats	
	Number of cells (10^9/L)	% of WBC	Number of cells (10^9/L)	% of WBC
WBC	9.55 (8.43-12.25)		10.20 (7.70-13.70)	
Monocytes	0.35 (0.30-0.46)	3.5 (3.0-4.5)	0.48 (0.35-0.66)	5.3 (3.9-6.0)*
Lymphocytes	8.25 (6.85-9.60)	79.0 (75.5-83.5)	6.44 (4.66-7.71)*	60.8 (56.0-66.63)*
Granulocytes	1.62 (1.43-2.23)	17.0 (13.5-19.5)	2.91 (2.10-4.90)*	34.0 (28.4-37.8)*

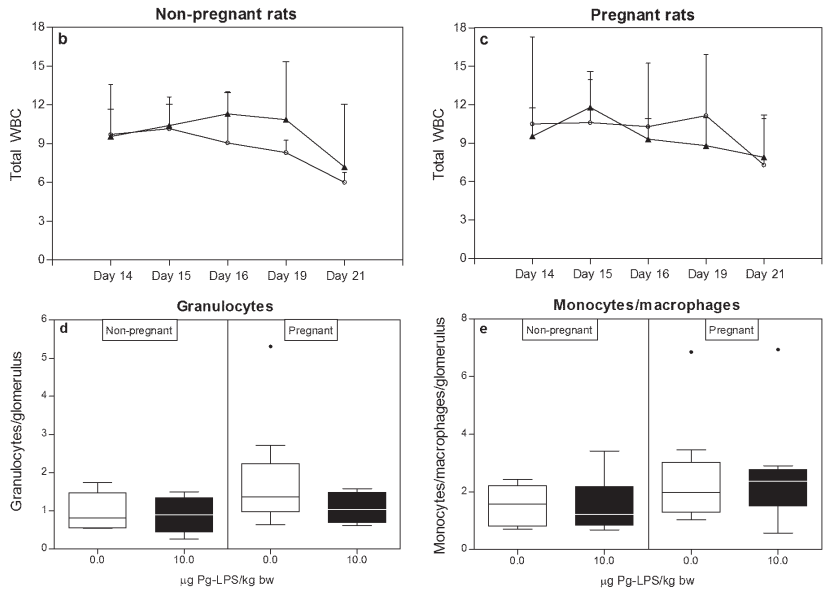

Figure 5. (**a**) Median and 25th and 75th percentiles of total and differential white blood cell counts (WBC) in non-pregnant and pregnant rats (gestational day 14), prior to infusion with saline or 10.0 µg Pg-LPS/kg bw; (**b, c**) median and 25th and 75th percentiles of total white blood cell (WBC) counts in non-pregnant (**b**) and pregnant (**c**) rats at day 14 (pre-infusion), and at days 15, 16, 19 and 21 of pregnancy (post-infusion days 1, 2, 5 and 7 respectively) after infusion of saline (closed triangles) or 10.0 µg Pg-LPS/kg bw (open dots); (**d**) median and 25th and 75th percentiles of number of granulocytes and (**e**) monocytes/macrophages per glomerulus in non-pregnant and pregnant rats, seven days after infusion with saline or 10.0 µg Pg-LPS/kg bw (gestational day 21 of pregnant rats). Error bars demonstrate 1.5 interquartile range and outlier values are represented as black dots. Open bars: saline infused control rats; black bars: Pg-LPS infused rats.

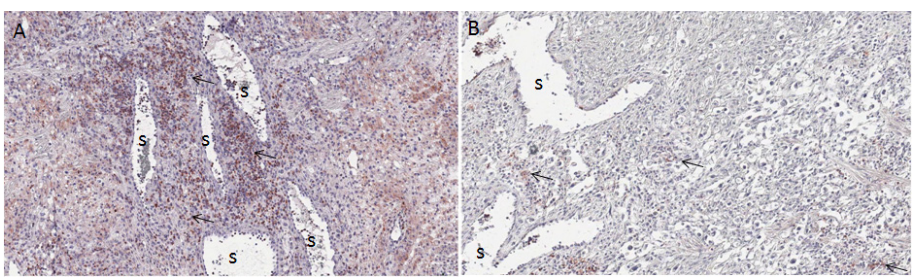

Figure 6. Photomicrographs of parts of the mesometrial triangle of a control pregnant rat at day 21 of pregnancy, stained for the presence of monocytes/macrophages (ED1; **a**) or for the presence of granulocytes (His48; **b**). ED1 positive cells were found throughout the mesometrial triangle, but especially around the spiral arteries (S). Less His48 positive cells were found in the mesometrial triangle, and they were found throughout the mesometrial triangle. S: spiral artery; arrows indicate positive cells.

Discussion

This study was set up to evaluate a causal relationship between Pg-LPS and pregnancy complications. Therefore, it was examined whether Pg-LPS infusion in pregnant rats could induce maternal hypertension, proteinuria and foetal or placental growth restriction and general inflammation. Rats were infused at day 14 of pregnancy, since this is in accordance with previous studies[24;29]. For these previous studies, this time point was chosen, since at day 14 of pregnancy the chorioallantoic placenta is fully developed, while the trophoblast invasion into the decidua and mesometrial triangle starts around that day[30;31]. Our results showed that Pg-LPS infusion in day 14 pregnant rats increased maternal systolic blood pressure, decreased placental weight and slightly decreased foetal weight (only in experiment 2) and also slightly increased the number of foetal resorptions, but did not induce albuminuria. Moreover, in the present study, no signs of placental or renal inflammation were seen, and also there were no signs of inflammation in the peripheral circulation (no increase in white blood cell counts or changes in white blood cell populations). Therefore, in this study we observed no generalised inflammatory response in pregnant rats after infusion of Pg-LPS. The effects of Pg-LPS appeared to be specific for pregnant rats, since hypertension was not induced in non-pregnant animals.

Our findings of increased blood pressure, decreased foetal weight and increased number of foetal resorptions may originate from the placenta as placental weight was dose dependently decreased by Pg-LPS infusion. The reason for the decreased placental weight remains unclear from this study. Since we did not observe local inflammation in the placental tissues, Pg-LPS may have had a direct effect on the placenta. The fact that placental weight was dose dependently decreased may support this suggestion. Pg-LPS is a Toll-like receptor 2 (TLR2) ligand[32] and placental trophoblast cells express TLR2[33]. It has been demonstrated in vivo and in vitro that activation of TLR2 receptors on trophoblast cells can evoke cell death pathways[34] and trophoblast apoptosis[35]. It can be hypothesised that the reduction in placental size may be associated with an increased resistance to blood flow in the placental arteries. Increased arterial resistance may result in reduced transport of oxygen and nutrients to the foetus and in some foetuses to foetal demise or death[23]. It is suggested that reduced placental perfusion in humans enhances the release of pro-inflammatory factors, such as cytokines, soluble endoglin (sEng), the soluble form of the vascular endothelial growth factor (VEGF) receptor (sFlt-1) and placental growth factor (PlGF). Although these factors may be involved in endothelial dysfunction, subsequently leading to hypertension[36], they are also involved in inducing generalised inflammation. Since in the present study there were no signs of systemic inflammation, it seems unlikely that the placenta released such pro-inflammatory factors into the maternal circulation.

It can be hypothesised that the increase in blood pressure in the present study may

have been a compensatory attempt to maintain perfusion in the affected placentas. Such an adaptive role for the foetus has also been suggested to occur in humans in the face of uteroplacental dysfunction[37]. Indeed, maternal hypertension during pregnancy has been associated with improved neonatal health[37;38]. The observation that antihypertensive therapy of mild-to-moderate hypertension during pregnancy appeared to impair intrauterine foetal growth further supports this hypothesis[39;40]. In the present study, even in the face of hypertension, foetal growth restriction or foetal death still occurred in some foetuses, suggesting that also other factors may play a role. Alternatively, it is also possible that the increased systolic blood pressure was the consequence of a direct effect of Pg-LPS on the endothelium, for it has recently been shown that exposure to Pg-LPS increases the sensitivity of contractile responses mediated by endothelin-1 (ET-1) in cultured rat coronary arteries[41]. Since ET-1 is a strong vasoconstrictor produced by endothelial cells that has been associated with hypertension during pregnancy in both animal and human studies[42], this may also be a possible mechanism by which Pg-LPS directly increased systolic blood pressure in pregnant rats in the present study.

Previously, our laboratory established a model for preeclampsia[24] by infusion of low doses of LPS (1.0 µg/kg bw) of *E. coli* in pregnant rats. These rats developed not only hypertension and a slightly decreased foetal weight, but in contrast to the present study, also proteinuria and generalised inflammation[24;29;43]. These differences in responses to both LPS species may be due to differences in signaling via TLRs. LPS from *E. coli* signals through TLR4, which leads to predominantly pro-inflammatory cytokine production[44]. Indeed, these animals showed a generalised inflammatory response[24;29;43]. The detrimental effects of this pro-inflammatory response induced by *E. coli* LPS were better observed after infusion of a slightly higher dose of this LPS (6.5 µg/kg bw), which induced septic-like signs, such as hypotension and maternal illness and increased numbers of foetal resorptions[24].

In contrast, Pg-LPS signals via TLR2[32], and in vitro stimulation of whole blood with Pg-LPS induced a lower pro-inflammatory cytokine production as compared with in vitro stimulation with *E. coli* LPS[45]. Interestingly, increasing the dose of Pg-LPS from 10.0 µg/kg bw to 50.0 µg/kg bw did not result in a further rise in blood pressure or foetal resorptions, a further decrease in foetal weight, or in other septic-like signs as compared with 10.0 µg/kg bw Pg-LPS. Since in vitro this Pg-LPS induced a dose dependent increase in cytokine production of monocytes[45], suggesting that also in this in vivo study higher Pg-LPS doses induced higher cytokine production. This lack of dose dependency, therefore, remains to be investigated.

Although various clinical and epidemiological studies suggest a relationship between periodontitis and pregnancy complications, there are not many animal or in vitro studies evaluating possible causal mechanisms. The results of the present study

indicate that Pg-LPS may be (one of) the causal mechanisms between periodontitis and adverse pregnancy outcomes. By affecting placental growth, Pg-LPS may be one of the contributing factors by which the periodontal infection causes unfavourable pregnancy outcomes including foetal demise and maternal hypertension. The results of the present study do not confirm a causal relationship between periodontitis and preeclampsia, for we did not observe albuminuria or inflammation. This study also shows that the effects of Pg-LPS appear relatively mild as compared with effects of *E. coli* LPS, suggesting that *E. coli* LPS may be more harmful for pregnancy than Pg-LPS.

In summary, this study showed that Pg-LPS infusion in pregnant rats increased maternal systolic blood pressure, impaired placental growth and slightly decreased foetal weight and also slightly increased numbers of foetal resorptions. Pg-LPS infusion did not induce albuminuria, a systemic maternal inflammatory response, or local inflammation in the kidneys or placentas. The present data provide a next step in the identification of the mechanism in the relationship between periodontitis and adverse pregnancy outcomes. The study undertaken suggests that Pg-LPS may be causally involved in some pregnancy complications, such as hypertension, foetal growth restriction, foetal death or miscarriage (in this rat model represented as foetal resorptions). However, a causal relationship of Pg-LPS with preeclampsia seems unlikely from the present study. Further research is therefore needed to identify the exact pathogenic mechanisms by which *P. gingivalis* or its products induce pregnancy complications, including placental and foetal growth restriction or maternal hypertension.

Acknowledgements

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Conflict of interest statement

The authors report no financial relationship related to any materials or products used in the present study.

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CHAPTER 5

Porphyromonas gingivalis and E. coli induce different cytokine production patterns in pregnant individuals

This chapter is an edited version of the manuscript:
Faas MM, Kunnen A, Dekker DC, Harmsen HJ, Aarnoudse JG, Abbas F, de Vos P, van Pampus MG. *Porphyromonas gingivalis* and *E. coli* induce different cytokine production patterns in pregnant individuals (submitted for publication, 2013)

Abstract

Objective: Pregnant individuals are more sensitive to various bacteria or their products. Pregnant individuals also respond differently to different bacteria or their products. Therefore, in the present study, we evaluated whether the increased sensitivity of pregnant individuals to bacterial products, and the different responses of pregnant individuals to different bacteria was associated with differences in whole blood cytokine production upon stimulation with bacteria or their products.

Study Design: Blood samples were taken from healthy pregnant and non-pregnant women and ex vivo stimulated with bacteria or LPS from *P. gingivalis* (Pg) or *E. coli* for 24 hrs. TNF-α, IL-1ß, IL-6, IL-12 and IL-10 were measured using a multiplex luminex system.

Results: We observed a generally lower cytokine production after stimulation with Pg bacteria or it's LPS as compared with *E. coli* bacteria. However, there was also an effect of pregnancy upon cytokine production: in pregnant women the production of IL-6 upon Pg stimulation was decreased as compared with non-pregnant women. After stimulation with *E. coli*, the production of IL-12 and TNF-α was decreased in pregnant women as compared with non-pregnant women.

Conclusion: Our results showed that cytokine production upon bacterial stimulation of whole blood differed between pregnant and non-pregnant individuals, showing that the increased sensitivity of pregnant individuals may be due to differences in cytokine production. Moreover, pregnancy also affected whole blood cytokine production upon Pg or *E. coli* stimulation differently. Thus, the different responses of pregnant individuals to different bacteria or their products may result from variations in cytokine production.

Introduction

Periodontal diseases are a group of diseases caused by inflammation and destruction of the supporting and investing structures of the teeth and the periodontal tissues[1]. The infection in the oral cavity can lead to systemic inflammation resulting in adverse medical outcomes. Indeed, associations between periodontal disease and cardiovascular disease (CVD)[2], stroke[3], glycemic control in diabetes[4] and rheumatoid arthritis[5] have been found. It has also become evident that periodontitis during pregnancy may result in adverse outcome; the presence of periodontitis during pregnancy has been associated with IUGR[6] or preterm birth[7]. Although there are many bacterial species present in the infected oral cavity, *P. gingivalis* (Pg) has most frequently been associated with systemic disease[8]. This is probably due to the fact that this bacterium has the capacity to disseminate into the peripheral circulation and cause inflammation at other sites[8].

The mechanism responsible for the association between periodontitis and pregnancy complications remains to be unraveled, but a route via the peripheral circulation to the placenta is likely to be involved[9;10]. Plausibly, also activation of the systemic inflammatory response by oral bacteria, such as Pg or their products, is involved. It is well known that pregnancy is a pro-inflammatory condition[11], with phenotypically activated monocytes and changes in monocyte function, such as cytokine production[12;13]. Therefore, it seems likely that during pregnancy the systemic inflammatory response to bacteria and their products is different as compared with this response in non-pregnant individuals. Indeed, pregnant individuals are much more sensitive to one of the *E. coli* products, lipopolysaccharide (LPS), than non-pregnant individuals[14]. For instance it has been shown that infusion of a low dose of *E. coli* LPS (1.0 µg/kgbw) induced hypertension and proteinuria in pregnant animals only; non-pregnant rats did not develop these signs[15].

Interestingly, infusion of a low dose of Pg-LPS into pregnant rats in identical circumstances induced hypertension, but not proteinuria. Moreover, while only slightly increased doses of *E. coli* LPS induced hypotension, maternal illness and resorption of most of the fetuses[15], increasing doses of Pg-LPS did not induce more severe effects than the low dose of Pg-LPS (Kunnen et al, submitted). This suggests that pregnant individuals are not only more sensitive to bacterial products, but also that the sensitivity of pregnant individuals to different bacteria or their products differs. In the present study, we evaluated whether the increased sensitivity of pregnant individuals to bacterial products, and the different sensitivities of pregnant individuals to different bacterial products was due to differences in cytokine production of leukocytes upon stimulation of whole blood with bacterial products. To this end, we compared cytokine production following stimulation of whole blood of pregnant and non-pregnant women with Pg or *E. coli* bacteria and their LPS and measured production of the pro-inflammatory cytokines TNF-α, IL-1β, IL-12 and IL-6 as well as the anti-inflammatory IL-10.

Materials and methods

Experimental design

To compare whole blood cytokine production in non-pregnant and pregnant women following stimulation with Pg or *E. coli* bacteria or their LPS, we stimulated whole blood of non-pregnant and pregnant women with bacteria of Pg or *E. coli* or their LPS. After 24 hrs. of stimulation, we measured the production of pro-inflammatory and anti-inflammatory cytokines in the plasma using a multiplex Luminex system.

Subjects

This study was approved by the local ethics committee and a written informed consent was obtained from each subject before participation. Participants (pregnant and non-pregnant, healthy Caucasian women between 20 and 40 years) were recruited from the Department of Obstetrics and Gynecology, University Medical Center Groningen or recruited from the hospital staff. Exclusion criteria for both groups were: smoking, hypertension, chronic diseases, flu-like symptoms or fever, treatment with antibiotics within 14 days prior to blood sampling or an DPSI score of 3+ or 4 after periodontal screening, which is indicative for destructive periodontal disease[16]. Furthermore, pregnant women were checked until the end of pregnancy and no pregnancy complications were observed.

Whole blood (10 ml; lithium-heparin vacutainer tube (Becton Dickinson, Rutherford, NJ)) was obtained by venous puncture from 16 primigravid women at 30 weeks of gestation (range 28-32 weeks) and from 15 nulligravid women with regular menstrual cycles (26-32 days) in their follicular phase (day 8-10), to minimize variations due to hormonal changes.

Bacteria

E. coli ATCC 25922 was grown on 5% sheep blood agar plates (Mediaproducts Groningen, The Netherlands) in air with 5%CO_2 at 37°C for 1 day. *P. gingivalis* ATCC 33277 (A.J. van Winkelhoff, Department of Oral Microbiology, Academic Center for Dentistry Amsterdam, The Netherlands) was grown on Brucella blood agar (Mediaproducts), supplemented with 5% sheep blood, 5 mg/L hemin and 1 mg/L menadione in an anaerobic chamber with 5%CO_2, 10%H_2 and 85%N_2 at 37°C. After 1 day (*E. coli*) or 4-7 days (Pg), one bacterial colony was inoculated in Todd-Hewitt-broth (BBL Microbiology Systems), supplemented with hemin (5 mg/L), menadione (5 mg/L) and glucose (2 mg/L) for one day (*E. coli*) or one week (Pg). The bacterial cultures were harvested by centrifugation at 2773 g for 10 minutes at 4°C. The pellet was washed twice in phosphate-buffered saline (PBS). The number of bacteria was evaluated by means of a microscope after gram-staining and resuspended in PBS at a concentration corresponding of approximately $1x10^8$ bacteria/ml and stored at -80°C.

Lipopolysaccharides

P. gingivalis LPS ATCC 33277 (Ultra-Pure, Cat.#: tlrl-pglps, Lot.#: 28-06-PGLPS, InvivoGen, San Diego, USA); *E. coli* LPS (055:B5, BioWhittaker, Walkersville MD, USA).

Stimulation of whole blood with bacteria and LPS

After sampling, 250 µl of blood was mixed with 250 µl of bacterial cultures of *E. coli* or Pg (final numbers: $5x10^7$ bacteria/ml). Furthermore, 250 µl of blood was mixed with 250 µl RPMI (Invitrogen, California, USA) and LPS (*E. coli* or Pg) was added (final concentration: 2 µg/ml). Negative controls were incubated in the absence of bacteria or LPS. Samples were incubated for 24h at 37°C in a 5%CO_2 humidified atmosphere. After stimulation, all samples were pipetted into 1.5 ml eppendorf tubes and centrifuged for 10 minutes at 316 g (4°C). The plasma was centrifuged again for 5 minutes at 1972 g (4°C) and frozen at -80°C.

Determination of plasma cytokine production

Cytokine levels in whole blood were measured using a Bio-PlexTM premixed cytokine assay, human 5-plex group I; cat. #: M50019PLCW, control 5016683 (Bio-Rad Laboratories, Hercules, USA), to measure TNF-α, IL-1β, IL-6, IL-10 and IL-12(p70), according to the manufacturers instruction manual. Raw data (mean fluorescence intensity, MFI) were analyzed using STarStation V2.3.

TLR labeling

Immediately after sampling, 500 µl of whole blood was mixed with 500 µl of RPMI and incubated with PerCp–labeled mouse-anti-human-CD14 (clone TüK4; Invitrogen Corporation, Breda, The Netherlands) together with FITC-labeled mouse-anti-human-TLR2 (clone TL2.1; eBioscience, Breda, The Netherlands) and PE-labeled mouse-anti-human-TLR4 (clone HTA 125; eBiosciences), or with anti-CD14 together with TLR2 and TLR4 isotype controls for 30 minutes at room temperature (RT) in the dark. After 5 minutes incubation with lysing buffer (Becton Dickinson, CA, USA) at RT in the dark, tubes were centrifuged (5 minutes at 467 g) and aspirated. After washing with washing buffer (PBS with 0.5% bovine serum albumin and 0.1% sodium azide), cells were fixed with 0.5% paraformaldehyde and kept at 4°C in the dark until flow cytometry, within 24h after labeling.

Flow Cytometry

Cells were analyzed by flow cytometer (FACS Calibur; Becton Dickinson, NJ, USA). For each individual, 100.000 leukocytes were acquired whilst life gating on leukocytes using forward and side-scatter characteristics. Data were saved for later analysis using Winlist 6.0 software (Verity Software House, Topsham, ME, USA).

During analyses a gate was set on the leukocytes in the forward-sidescatter plot. This gate was copied to a sidescatter-CD14 plot, in which monocytes (CD14 positive cells), granulocytes (CD14 negative cells with high SSC) and lymphocytes (CD14 negative cells with low SSC) were gated. Total numbers of monocytes, granulocytes and lymphocytes were derived by multiplying the percentage of the subpopulations with the total WBC count (microcell counter model Sysmex pocH-100i Haematology Analyser, Sysmex Corp., Kobe, Japan). Thereafter, CD14 positive cells were copied to a TLR2/TLR4 plot. Using the isotype control sample, gates were set in the TLR2/TLR4 plot so that at least 99% of the isotype controls were negative for TLR2/TLR4 expression. This gate was then used to identify the percentages of TLR4/TLR2 double positive, TLR2 single and TLR4 single positive monocytes as well as their mean fluorescent intensity (MFI), in the antibody incubated samples.

Data analysis

All figures expressed individual results (line: mean or median, depending on normality of the data). Normality of the data was tested using the Kolmogorov-Smirnov test. In the blood stimulation experiments, effects of the reproductive state (non-pregnant vs. pregnant) or effects of the bacteria or LPS species (*E. coli* vs. Pg) were tested using two-way ANOVA followed by Bonferroni post-tests. In case data were not normally distributed, before using the two-way ANOVA, data were log transformed, which led to normal distribution of data. For data on number of WBC and the differential cell counts and data on TLR expression, differences between pregnant and non-pregnant women in were evaluated using the Student's *t*-test. In all cases, the significance level was $p<0.05$.

Results

Whole blood cytokine production following bacterial stimulation

Figure 1 shows that for all cytokines tested, in pregnant and in non-pregnant women, *E. coli* bacteria induced a stronger cytokine production as compared with Pg bacteria (Two-way ANOVA and Bonferroni posttest, $p<0.05$). An effect of pregnancy was also observed: the concentration of IL-12 following *E. coli* stimulation was significantly lower in pregnant blood as compared with non-pregnant blood ($p<0.05$). The concentration of IL-6 following Pg stimulation was significantly lower ($p< 0.05$) in pregnant blood vs. non-pregnant blood.

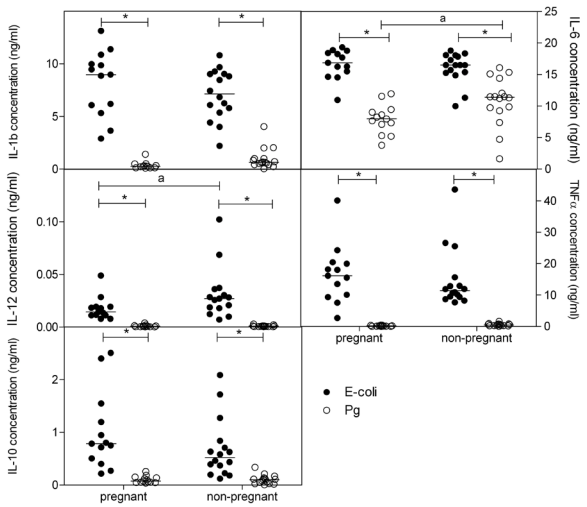

Figure 1: Cytokine concentrations following stimulation with bacteria.
Concentrations (ng/ml) of IL-1β, IL-6, IL-12, TNF-α and IL-10 in plasma of pregnant and non-pregnant women following stimulation of whole blood with *E. coli* (black dots) or *P. gingivalis* (Pg) (open dots) bacteria ($5*10^7$ bacteria/ml) for 24 hr. *significantly different from *E. coli* (two-way ANOVA followed by Bonferroni post-tests, $p<0.05$)) a: significantly different from pregnant women after the same stimulation (two-way ANOVA followed by Bonferroni post-tests, $p<0.05$)

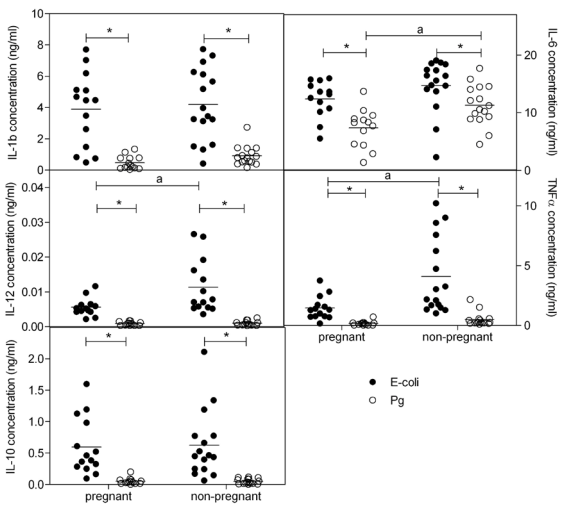

Figure 2: Cytokine concentrations following stimulation with LPS.
Concentrations (ng/ml) of IL-1β, IL-6, IL-12, TNF-α and IL-10 in plasma of pregnant and non-pregnant women following stimulation of whole blood with *E. coli* (black dots) or *P. gingivalis* (Pg) (open dots) LPS (2 µg/ml) for 24 hr. *significantly different from *E. coli* (two-way ANOVA followed by Bonferroni post-tests, $p<0.05$)) a: significantly different from pregnant women after the same stimulation (two-way ANOVA followed by Bonferroni post-tests, $p<0.05$)

Whole blood cytokine production following LPS stimulation

Also stimulation of blood of pregnant and non-pregnant women with *E. coli* LPS induced significantly higher production of all cytokines tested as compared with Pg-LPS (Two-way ANOVA and Bonferroni posttest, $p<0.05$). Moreover, concentrations of IL-12 and TNF-α after stimulation with *E. coli* LPS were significantly lower in pregnant as compared with non-pregnant women. The concentration of IL-6 was significantly lower in pregnant vs. non-pregnant women following stimulation with Pg-LPS (fig 2).

Ratio of IL-12/IL-10, TNF-α/IL-10, IL-6/IL-10

Stimulation with *E. coli* bacteria resulted in a significantly higher IL-12/IL-10, TNF-α/IL-10 ratio and a significantly lower IL-6/IL-10 ratio as compared with stimulation with Pg bacteria (fig 3A) in both pregnant and non-pregnant women. Pregnant women showed a decreased IL-12/IL-10 ratio after stimulation with *E. coli* bacteria (fig 3A) and a decreased IL-6/IL-10 ratio following Pg bacterial stimulation as compared with non-pregnant women.

After LPS stimulation, we observed a higher IL-12/IL-10 ratio after *E. coli* LPS stimulation vs. Pg-LPS in blood of pregnant women and a lower IL-6/IL-10 ratio after *E. coli* LPS stimulation vs. Pg-LPS stimulation in blood of both pregnant and non-pregnant women. The IL-12/IL-10 ratio was decreased in pregnant vs. non-pregnant women for both types of LPS, while only for Pg-LPS the TNF-α/IL-10 and the IL-6/IL-10 ratio was decreased in pregnant vs. non-pregnant women (fig 3B).

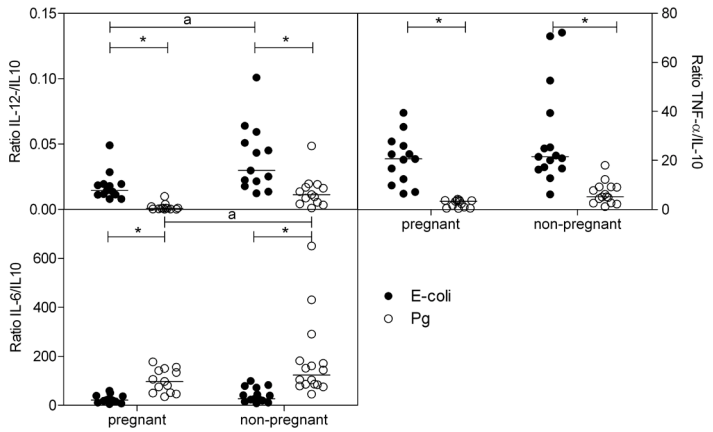

Figure 3A: Pro-inflammatory/anti-inflammatory cytokine ratio following stimulation with bacteria. Ratio of IL-12/IL-10, TNF-α/IL-10 and IL-6/IL-10 cytokine production in plasma of pregnant and non-pregnant women following stimulation of whole blood *E. coli* (black dots) or *P. gingivalis* (Pg) (open dots) bacteria ($5*10^7$ bacteria/ml) for 24 hr. *significantly different from *E. coli* (two-way ANOVA followed by Bonferroni post-tests, $p<0.05$)). a: significantly different from pregnant women after the same stimulation (two-way ANOVA followed by Bonferroni post-tests, $p<0.05$)

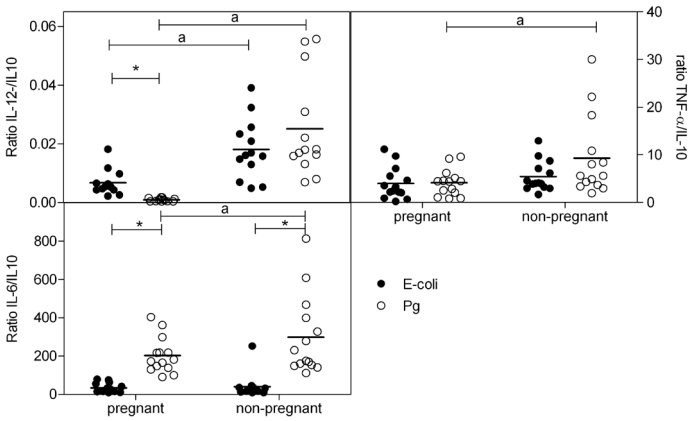

Figure 3B: Pro-inflammatory/anti-inflammatory cytokine ratio following stimulation with LPS. Ratio of IL-12/IL-10, TNF-α/IL-10 and IL-6/IL-10 cytokine production in plasma of pregnant and non-pregnant women following stimulation of whole blood *E. coli* (black dots) or *P. gingivalis* (Pg) (open dots) LPS (2 µg/ml) for 24 hr. *significantly different from *E. coli* (two-way ANOVA followed by Bonferroni post-tests, $p<0.05$)). a: significantly different from pregnant women after the same stimulation (two-way ANOVA followed by Bonferroni post-tests, $p<0.05$)

Changes in white blood cells counts and TLR2 and TLR4 expression in pregnant women

As cytokine production in the plasma may depend on the number of leukocytes, we measured WBC counts and percentages of leukocyte subsets in the blood samples (Table 1). A significant increase in total number of WBC, monocytes and granulocytes was seen during pregnancy as compared with the follicular phase ($p<0.05$, Student's *t*-test).

Table 1: total white blood cell count and differential cell counts

	WBC Count (*10^9/L) (mean±SEM)	Granulocyte count (*10^9/L) (mean±SEM)	Monocyte count (*10^9/L) (mean±SEM)	Lymphocyte count (*10^9/L) (mean±SEM)
Pregnant	9.96±0.62*	7.02±0.56*	0.62±0.07*	2.31±0.17
Non-pregnant	5.69±0.21	2.89±0.25	0.35±0.03	2.44±0.15

*: significantly increased vs non-pregnant women (Student's *t*-test, $p<0.05$)

Since TLR2 and TLR4 are the main TLRs recognizing bacteria and LPS[17], we measured expression of these receptors on monocytes, the most important cells responsible for bacteria and LPS recognition. The percentage of TLR2+ monocytes decreased in pregnant vs. non pregnant women (fig 4A; Student's *t*-test, $p<0.05$), while the mean fluorescent intensity (MFI), a measure for expression of TLR2 per cell, was not affected

by pregnancy. The percentage TLR4+ monocytes and TLR4 MFI of monocytes was not different between pregnant and non-pregnant women (fig 4B).
The percentage of double positive cells was also not affected by pregnancy (56.41±4.42 in pregnant vs. 57.85±4.48 in non-pregnant women; not shown).

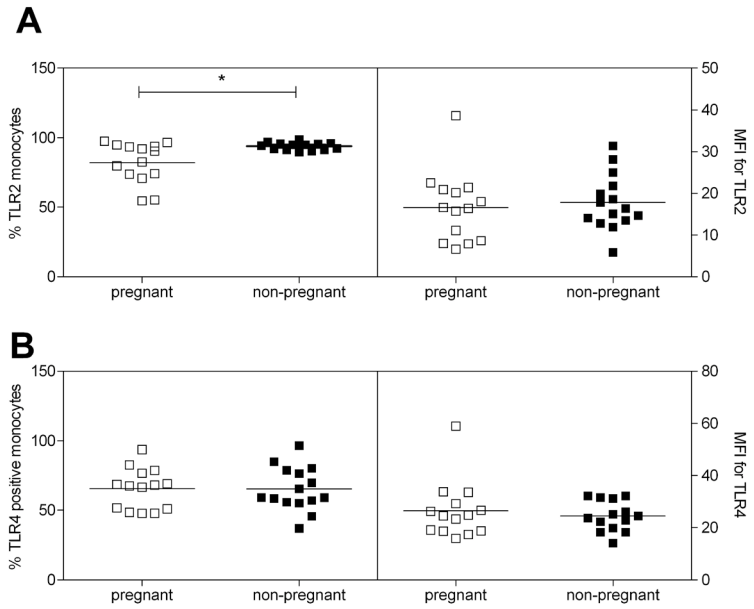

Figure 4: Expression of TLR2 and TLR4 in pregnant and non-pregnant women.
Expression of TLR2 and TLR4 on monocytes of pregnant (open squares) and non-pregnant women (black squares). A: Percentage of TLR2 positive monocytes (left graph) and mean fluorescent intensity of TLR2 staining of monocytes (right graph). B: Percentage of TLR4 positive monocytes (left graph) and mean fluorescent intensity of TLR4 staining of monocytes (right graph). *: significantly increased vs pregnant women (student's *t*-test, $p<0.05$)

Comment

The present study was conducted to evaluate the effect of pregnancy and different bacteria and their products on leukocyte cytokine production. We stimulated whole blood of pregnant and non-pregnant women with bacteria or LPS from *E. coli* or Pg. There was a generally lower cytokine production after stimulation with Pg bacteria or it's LPS as compared with *E. coli* bacteria or it's LPS in both non-pregnant and pregnant women. We also observed an effect of pregnancy upon cytokine production. In pregnant women the production of IL-6 upon Pg stimulation was decreased as compared with non-pregnant women, while the production of IL-12 and TNF-α was decreased in pregnant women as compared with non pregnant women following stimulation with *E. coli*. This illustrates that pregnancy affects cytokine responses upon

Pg or *E. coli* stimulation differently and suggests that the varying responses during pregnancy upon different bacteria or their products may result from differences in cytokine production. The increased sensitivity of pregnant women to bacteria or their products may also result from differences in cytokine production.

We found a marked lower cytokine production and a relatively higher production of pro-inflammatory cytokines induced by Pg bacteria/LPS in comparison with *E. coli* bacteria/LPS in both pregnant and non-pregnant women. An important mechanism by which a decreased cytokine response upon LPS/bacterial stimulation could be explained is by decreased expression of pattern recognition receptors (PPR), amongst which Toll-like receptors (TLR) are the best studied[18]. These TLR recognize so-called DAMPs (danger-associated molecules)[19]. PAMPs (pathogen-associated-molecules), are of subtype of DAMPS; they arise from pathogens, and alarm an individual to intruding pathogens[19]. LPS or other bacterial factors are PAMPs which are recognized by TLR2 and TLR4[17]. As TLR2 is involved in recognition of Pg-LPS[20;21] and TLR4 in recognition of *E. coli* LPS[21], differences in expression between TLR2 and TLR4 on monocytes may result in different cytokine production following stimulation with these bacteria/LPS. However, despite the lower cytokine production after Pg bacteria/LPS, TLR2 is higher expressed by monocytes as compared with TLR4. Differences in TLR expression could also explain differences in responses of pregnant vs. non-pregnant women to Pg or *E. coli* LPS. We found a decreased expression of TLR2 on monocytes of pregnant vs. non-pregnant women, with no changes in TLR4 expression. Although production of some cytokines were decreased during pregnancy after stimulation with Pg-LPS, this was not the case for all cytokines. Further research is warranted to evaluate the role of TLRs on cytokine production in pregnant women.

The finding that cytokine production after stimulation with Pg bacteria/LPS is generally lower as compared with stimulation with *E. coli* bacteria/LPS in non-pregnant women is in line with previous studies[22;23]. Our study for the first time shows these differences in pregnant women. Such lower cytokine production and lower pro-inflammatory cytokine ratio following stimulation with Pg-LPS, as compared with *E. coli* LPS, may be involved in the in vivo differences in responses of pregnant women or animals to these LPS species: while *E. coli* LPS induces a preeclampsia-like syndrome[15], Pg LPS only induced hypertension [Kunnen et al, accepted for publication, Oral Diseases 2013]. Apparently, a preeclampsia-like syndrome is induced by pro-inflammatory cytokines, which is in line with the fact that the pro-inflammatory TNF-α also induced a preeclampsia-like syndrome[24]. The differences in cytokine production following stimulation with Pg bacteria vs. *E. coli* bacteria may suggest that pregnant women do also respond differently to these bacteria. Infections with *E. coli* bacteria usually result in maternal symptoms of illness, such as fever[25;26]. Although it has been shown that Pg bacteria can disseminate into the circulation, i.e. they can be found in the placenta[9;10],

such dissemination does not result in maternal illness. The present study suggests that these different maternal responses towards *E. coli* infection and Pg infection may (partly) result from differences in cytokine production following recognition of the bacteria. However, other mechanisms, such as differences in numbers of circulating bacteria between *E. coli* and Pg infection, may also play a role.

The differences in IL-6 production between the two strains are smaller as compared with the other cytokines. This is reflected in an increased IL-6/IL-10 ratio following Pg bacteria/LPS stimulation as compared with *E. coli* bacteria/LPS stimulation. These results are in line with the fact that IL-6 plays an important role in periodontal disease. IL-6 is an important cytokine with diverse functions. It regulates the immune response and leukocyte recruitment[27], but can also affect bone formation[28]. It has also been shown that IL-6 has potent anti-inflammatory properties, as it can inhibit the production of TNF-α[29] and can increase the production of IL-10 and IL-1ra[30]. Therefore the relatively high production of IL-6 induced by stimulation with Pg bacteria or LPS may, next to the relatively low overall cytokine production, be involved in the different response of individuals to these bacteria or its LPS.

Interestingly, despite the fact that pregnant women are much more sensitive to LPS, the production of cytokines following LPS (of both species) stimulation is either similar or decreased in pregnant women as compared with non-pregnant women. This suggests that pregnant women may be more sensitive to the effects of these cytokines. This is in line with earlier results from our lab[31]. If results would have been presented as amount of cytokines per monocyte, the differences would even be more extreme (results not shown), since the number of monocytes is increased in blood of pregnant women. Thus, as has also been shown previously[13], monocytes of pregnant women produce less cytokines upon a similar LPS or bacterial stimulus than monocytes of non-pregnant women. Such a decreased production of cytokines by pregnant monocytes may be due to their increased activational status: monocytes of pregnant women show increased CD14, CD11b and CD64 expression and decreased CD62L expression[12]. This may result in an endotoxin tolerant state, similar to the "endotoxin tolerance" seen in monocytes from septic patients[32].

In summary, the generally lower production of cytokines as well as the decreased pro-inflammatory ratio after Pg stimulation vs. *E. coli* stimulation in pregnant women may be responsible for the differences in the in vivo response upon the bacteria and their products in these individuals. Although pregnant women are extremely sensitive to LPS, the production of IL-12, TNF-α and IL-6 upon stimulation with bacteria or LPS were decreased, suggesting that pregnant women are more sensitive to these cytokines. The mechanism of decreased cytokine production during pregnancy requires further investigation.

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CHAPTER 6

Cytokine production induced by non-encapsulated and encapsulated Porphyromonas gingivalis strains

This chapter is an edited version of the manuscript:
Kunnen A, Dekker DC, van Pampus MG, Harmsen HJ, Aarnoudse JG, Abbas F, Faas MM. Cytokine production induced by non-encapsulated and encapsulated *Porphyromonas gingivalis* strains. *Arch Oral Biol.* 2012 Nov;57(11):1558-66

Abstract

Objective: Although the exact reason is not known, encapsulated gram-negative *Porphyromonas gingivalis* strains are more virulent than non-encapsulated strains. Since difference in virulence properties may be due to difference in cytokine production following recognition of the bacteria or their products by the host inflammatory cells, we compared cytokine production following stimulation with bacteria or lipopolysaccharides (LPS) of a non-encapsulated and an encapsulated *P. gingivalis* strain (K^- and K1).

Design: Tumour necrosis factor-alpha (TNF-α) production following stimulation of the cell-line Mono Mac 6 with bacteria or LPS of both *P. gingivalis* strains was determined using flow cytometry. Furthermore, we investigated the effects of the two *P. gingivalis* strains or their LPS on TNF-α and Interleukin (IL-1β, IL-6, IL-12 and IL-10) production in whole blood using Luminex. In both experiments, *Escherichia coli* bacteria and LPS were used as a reference.

Results: Both *P. gingivalis* strains induced lower cytokine production than *E. coli* with the exception of IL-6. *P. gingivalis* K1 bacteria elicited a higher overall cytokine production than *P. gingivalis* K^-. In contrast, *P. gingivalis* K1 LPS stimulation induced a lower cytokine production than *P. gingivalis* K^- LPS.

Conclusions: Our findings suggest that the encapsulated *P. gingivalis* K1 bacteria induce higher cytokine production than the non-encapsulated *P. gingivalis* K^-. This was not due to its LPS. The stronger induction of cytokines may contribute to the higher virulence of *P. gingivalis* K1.

Introduction

Periodontitis is a multifactorial, polymicrobial infection of the tissues surrounding the teeth, caused by a mixed microflora consisting of gram-negative and gram-positive micro-organisms. It is a chronic inflammatory disease involving complex interactions between the micro-organisms and immune response of the host and is characterised by collagen destruction and alveolar bone resorption[1].

An important gram-negative strict anaerobic micro-organism associated with periodontal breakdown and with the capacity to modulate inflammatory responses is *P. gingivalis*[2;3]. Several encapsulated and non-encapsulated serotypes of *P. gingivalis* have been described[4-6]. Encapsulated *P. gingivalis* serotypes are more virulent in experimental infections and cause a more invasive type of infection, whereas non-encapsulated *P. gingivalis* cause a more localised infection[7]. The exact reason for the higher virulence of encapsulated strains is not known, but may result from different cytokine production following recognition of the encapsulated or non-encapsulated bacteria by the host immune system[8;9]. *P. gingivalis* strains contain a variety of components on their cell surface, like lipopolysaccharides (LPS), lipoproteins and fimbriae, as well as capsular components, which may activate inflammatory cells, such as monocytes, and induce cytokine production[2]. Especially LPS is known as a potent stimulator of the host inflammatory response[10] and is thought to be a main virulence factor[2]. Therefore, we hypothesised that encapsulated and non-encapsulated *P. gingivalis* do induce different production of cytokines upon stimulation of inflammatory cells and that this may be due to their LPS.

Monocytes are the main regulators of the inflammatory response[11] by their ability to recognise bacteria and their products by pattern recognition receptors (PRRs), like Toll-like receptors (TLRs) and NOD-like receptors (NLRs)[12]. Upon recognition of bacteria or their products by these PRRs, monocytes start producing cytokines[13]. In the present study, we investigated whether encapsulated and non-encapsulated *P. gingivalis* strains induced different cytokine production in monocytes. First we used a monocyte cell line, Mono Mac 6 (MM6), to study whether there are differences in cytokine responses of monocytes per se to the encapsulated and non-encapsulated *P. gingivalis* strains. We used TNF-α as a marker of cytokine production, since TNF-α is the most important regulatory pro-inflammatory cytokine, which is first produced by monocytes/macrophages upon bacterial or LPS stimulation[14]. Since this experiment showed different TNF-α responses to especially the LPS of the two *P. gingivalis* strains, in a second experiment, we incubated whole blood to simulate the natural environment[15], with bacteria or LPS and measured production of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-12 and the anti-inflammatory cytokine IL-10. In both experiments, we used *E. coli* bacteria or its LPS as a reference, since this species is known to be a strong inducer of pro-inflammatory cytokines[16;17].

Materials and methods

Experimental design

Experiment 1: The first aim was to compare the pro-inflammatory properties of *P. gingivalis* K^-, *P. gingivalis* K1 and *E. coli* bacteria on monocytes per se. Therefore, we first evaluated the production of TNF-α after stimulation of MM6 cells[18] with the bacteria using flow cytometry. To study whether differences in virulence are the result of differences in pro-inflammatory potency of their LPS, we also determined TNF-α production by MM6 cells after stimulation with LPS of *P. gingivalis* (K^- and K1) as well as with *E. coli* LPS.

Experiment 2: Since in experiment 1 we found differences in TNF-α production by MM6 cells after stimulation with the LPS of the two *P. gingivalis* strains, we continued to study the production of other pro- and anti-inflammatory cytokines, now using whole blood, which simulates the in vivo situation better. For this experiment, whole blood of 15 healthy individuals was stimulated with bacteria as well as with LPS of *P. gingivalis* K^-, *P. gingivalis* K1 and *E. coli*. The production of TNF-α, IL-1β, IL-6, IL-12 and IL-10 was measured using the Luminex system.

Bacterial strains and growth conditions

E. coli ATCC 25922 was grown on 5% sheep blood agar plates (Mediaproducts, Groningen, The Netherlands) in air with 5% carbon dioxide (CO_2) at 37°C for 1 day. Bacterial strains of *P. gingivalis* ATCC 33277 (K^-), which has been shown to be non-encapsulated[19] and *P. gingivalis* W50 (K1), which has been shown to have a capsule[6;20], were generously provided by A.J. van Winkelhoff (Department of Oral Microbiology, Academic Center for Dentistry Amsterdam, The Netherlands). *P. gingivalis* K^- and K1 were grown on Brucella blood agar (Mediaproducts, Groningen, The Netherlands), supplemented with 5% sheep blood, 5 mg/l hemin and 1 mg/l menadione in an anaerobic chamber with an atmosphere of 5% CO_2, 10% H_2 and 85% N_2 at 37°C. After 4-7 days, one bacterial colony per strain was incubated in Todd-Hewitt broth (BBL Microbiology Systems), supplemented with hemin (5 mg/l), menadione (5 mg/l) and glucose (2 g/l) for one week. The bacterial cultures were harvested by centrifugation at 2773 × *g* for 10 minutes at 4°C. The supernatant was decanted and the bacterial pellet was washed twice in phosphate-buffered saline (PBS). The number of bacteria was evaluated by means of a microscope after gram-staining and resuspended in PBS at a number corresponding to approximately $1x10^8$ bacteria/ml. All cultures were stored at –20°C until used.

Lipopolysaccharides derived from *E. coli* 055:B5, (BioWhittaker, Walkersville, MD, USA); *P. gingivalis* ATCC 33277 (Ultra-Pure, Cat. #: tlrl-pglps, Lot. #: 28-06-PGLPS, InvivoGen, San Diego, USA) and *P. gingivalis* W50, a generous gift from M.A. Curtis (The Institute of Cell and Molecular Science; Barts and The London School of Medicine and Dentistry, UK) were used.

Cell line and culture

The MM6 cell line was cultured in RPMI (Complete Roswell Park Memorial Institute) 1640 medium (Invitrogen, California, USA) with 10% foetal calf serum (FCS), supplemented with 1% L-glutamin, 1% Na-pyruvate, 0.1% BME (2β-mercaptoethanol), 0.6% gentamycin sulfate and 0.05% fungizone (amfotericine B). Cell-suspensions were cultured at 37°C with a 5% CO_2 humidified atmosphere and diluted 1:5 in the culture medium for every 3-4 days.

Experiment 1: TNF-α production after stimulation of MM6 cells

Before stimulation, MM6 cells were counted and diluted in RPMI to a cell concentration of $4x10^9$ cells/l. $2x10^8$ MM6 cells/l were supplemented with 9% FCS and 2 ng/ml monensin (Sigma Aldrich, St. Louis, MO, USA) to inhibit intracellular transport of TNF-α through the ER-Golgi complex[21]. Immediately prior to use, the stocks of $1x10^8$ bacteria/ml of *E. coli*, *P. gingivalis* K^- and *P. gingivalis* K1 were thawed and used undiluted or diluted in PBS (10, 100 and 1000 times). 250 µl of bacterial suspensions was added to 50 µl of the MM6 suspension to reach a final bacterial concentration in the tubes of $8.3x10^7$, $8.3x10^6$, $8.3x10^5$ and $8.3x10^4$ bacteria/ml respectively. All cultures were incubated for 4 h at 37°C in a 5% CO_2 humidified atmosphere. Similarly, MM6 cells were incubated with increasing concentrations (0.005, 0.05, 0.25, 0.5, 1.0, 1.5 and 2.5 µg/ml) of *P. gingivalis* K^- LPS, *P. gingivalis* K1 LPS or with *E. coli* LPS. For negative control (unstimulated MM6 cells), MM6 cells were incubated in the absence of bacterial or LPS challenge.

Sample labelling

After incubation, 200 µl of both stimulated and unstimulated MM6 cells were fixed in 1 ml 2% paraformaldehyde (PFA) in PBS for 5 minutes. Then, after centrifugation at 467 × *g* for 5 minutes, the pellet was resuspended in 1 ml 0.1% saponin solution (Sigma Aldrich, St. Louis, MO, USA) in washing buffer [PBS with 0.5% bovine serum albumin (BSA) and 0.1% sodium azide] for 5 minutes in order to permeabilise the MM6 cells. After centrifugation (5 minutes at 467 × *g*) and aspiration, the cells were incubated with PE-labelled mouse-anti-human TNF-α antibody (BD Pharmingen, San Diego, USA), 1:10 diluted with 0.1% saponin solution in washing buffer for 30 minutes at room temperature in the dark. The MM6 cells were then washed again with 1 ml 0.1% saponin solution in washing buffer, and after centrifugation and aspiration, the cells were fixed with 200 µl 0.5% PFA in PBS and were kept in the dark at 4°C until measured by flow cytometry, within 24 h after labelling.

Flow cytometry

MM6 cells were analysed using the FACS Calibur flow cytometer (Becton Dickinson, NJ, USA). Flow cytometry results were analysed using Winlist 6.0 software (Verity Software House, Topsham, ME, USA).

Intracellular cytokines

Five thousand MM6 cells were acquired by live gating on the total MM6 population using forward- and sidescatter characteristics. This gate was copied to a single parameter histogram, to determine intracellular TNF-α production of the MM6 cells. Using the unstimulated control sample, a linear gate was set in the histogram so that at least 99% of the unstimulated MM6 cells were negative for TNF-α production. This gate was then copied to the histogram for stimulated MM6 cells. The percentage of positive cells was evaluated from the histogram of the stimulated cells. Results are expressed as percentage of TNF-α positive cells.

Experiment 2: cytokine production after stimulation of whole blood

Whole blood samples were obtained by venous puncture from 15 healthy females. Protocols for this study were approved by the local ethics committee and a written informed consent was obtained from each subject before participation. Since males and females do respond differently to endotoxin[22], in order to obtain a homogenous study-population, only Caucasian women between 20 and 40 years of age, with no known systemic diseases, were included in this study. Moreover, female hormonal fluctuations during the different phases of the ovarian cycle influence the sensitivity of monocytes to endotoxin[23], therefore, for all individuals, blood was drawn in the follicular phase of the menstrual cycle. Immediately after blood sampling, all participants were submitted to a periodontal examination by a certified dental hygienist (A.K.) using the Dutch Periodontal Screening Index (DPSI) to establish the periodontal condition[24]. To avoid the risk of including participants with a generalised inflammatory response due to periodontitis, we excluded subjects with a DPSI score of 3+ or 4, which is indicative for destructive periodontal disease[24]. Blood samples (10 ml) were collected in vacutainer tubes containing lithium heparin (Becton Dickinson, Rutherford, NJ, USA). The stock bacterial cultures were thawed. Immediately after sampling, 250 µl of undiluted stock bacterial cultures of *E. coli*, *P. gingivalis* K^- or *P. gingivalis* K1 were mixed with 250 µl of heparinised blood (final numbers: $5x10^7$ bacteria/ml). Furthermore, 250 µl of heparinised whole blood was mixed with 250 µl RPMI and LPS of *E. coli, P. gingivalis* K^- or *P. gingivalis* K1 was added (2 µg/ml). Negative controls were incubated in the absence of bacterial or LPS challenge under similar circumstances. All samples were incubated for 24 h at 37°C in a 5% CO_2 humidified atmosphere. After stimulation, all samples were pipetted into 1.5 ml Eppendorf tubes and centrifuged for 10 minutes at 316 × *g* (4°C). The plasma was then pipetted into new 1.5 ml Eppendorf tubes and centrifuged again for 5 minutes at 1972 × *g* (4°C). Supernatants were frozen at −80°C until cytokine concentrations were measured using Luminex.

Determination of extracellular cytokine production in whole blood

Cytokine levels in whole blood were measured using a Bio-Plex™ premixed cytokine

assay, human 5-plex group I; Cat. #: M50019PLCW, control 5016683 (Bio-Rad Laboratories, Hercules, USA). This customised kit simultaneously measured human TNF-α, IL-1β, IL-6, IL-10 and IL-12 (p70). Standard curves for each cytokine were generated using the reference cytokine concentrations supplied in this kit. Assay buffer, plasma and duplex standards were pipetted into the wells according to the manufacturers' instruction manual. In brief, after prewetting the wells of a 96-well filter plate with assay buffer, 50 µl of coupled beads were added to the wells and washed twice with assay buffer using a vacuum manifold (Millipore, MA, USA). 50 µl of 1:3 diluted plasma (sample diluent supplied in the kit) and standards were pipetted into the wells and incubated for 30 minutes with the coupled beads. The wells were then washed three times and 25 µl of detection antibody was subsequently added. After 30 minutes incubation, the wells were again washed three times and incubated for 10 minutes with 50 µl streptavidin-PE. After 10 minutes of incubation, the wells were washed three times in order to remove the unbound streptavidin-PE. Finally, 125 µl of assay buffer was added to each well after which the beads were analysed using the Luminex LX100™ multiplex assay detection system. Raw data (mean fluorescence intensity, MFI) were analysed using STarStation V2.3.

Data analysis

Statistical analysis was performed using GraphPad Prism 5 for Windows (Graphpad Software, San Diego, CA, USA). Normality tests were performed using the Shapiro-Wilk test. Values were given as mean ± standard error of the mean (SEM) for normally distributed variables. Not normally distributed data were presented as box and whisker plots showing the median values, interquartile and full ranges of value. Outliers were defined as data points greater than 1.5 the interquartile range from the median value. To evaluate differences between the dose response curves of the various bacterial and LPS stimulations, we calculated the area under the curve (AUC). Statistical comparisons between the AUCs after the various bacterial and LPS stimulations were performed using the unpaired Student's *t*-test with Bonferroni's correction. Differences in cytokine concentrations in plasma after stimulation with the different bacterial species as well as after the different LPS stimulations were evaluated by using Wilcoxon's Signed Rank test with Bonferroni's correction. Pro- or anti-inflammatory ratios were calculated by dividing the pro-inflammatory cytokine production (TNF-α and IL-12) by the anti-inflammatory cytokine production (IL-10). The ratios between the various bacterial and LPS stimulations were evaluated by using Wilcoxon's Signed Rank test with Bonferroni's correction. For all experiments, a *p*-value of <0.05 was accepted as statistically significant.

Results

Experiment 1: TNF-α production after stimulation of MM6 cells

MM6 cells exposed to only medium showed no TNF-α producing MM6 cells. Stimulation with increasing numbers of bacteria of *P. gingivalis* K^- and *P. gingivalis* K1 showed a dose-dependent increase in the percentage of TNF-α positive monocytes (Fig. 1(a)). Differences in dose response curves after the various bacterial stimulations were calculated using the AUC (Fig. 1(a), inset). A significantly higher AUC was observed after stimulation with bacteria of *E. coli* as compared with *P. gingivalis* K^- and *P. gingivalis* K1 ($p<0.05$). No differences in the AUC between *P. gingivalis* K^- and *P. gingivalis* K1 stimulation were observed.

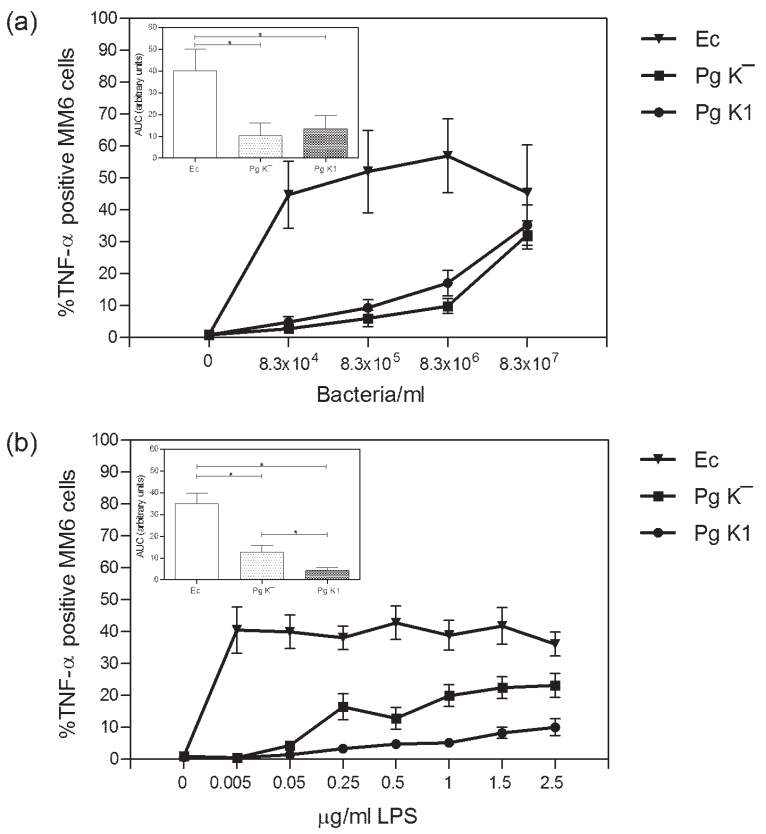

Fig. 1 – (a) Mean ± SEM percentages of TNF-α positive MM6 cells after stimulation with increasing numbers of bacteria from *P. gingivalis* K^- (Pg K^-) or *P. gingivalis* K1 (Pg K1), with *E. coli* (Ec) as reference strain and (b) mean ± SEM percentages of TNF-α positive MM6 cells after stimulation with increasing concentrations of LPS from Pg K^-, Pg K1 and from Ec as reference strain. Inserts: mean area under the curve (AUC) ± SEM of TNF-α positive MM6 cells after stimulation with increasing numbers of bacteria from Pg K^-, Pg K1 and Ec, or with increasing concentrations of LPS from Pg K^-, Pg K1 and Ec. (*) Significantly different (unpaired Student's t-test with Bonferroni's correction; $p<0.05$).

The percentages of TNF-α positive MM6 cells increased after stimulation with 0.005 µg/ml *E. coli* LPS, and then remained constant following stimulation with higher concentrations (Fig. 1(b)). Stimulation with increasing concentrations of *P. gingivalis* K^- LPS and K1 LPS induced a dose-dependent increase of TNF-α positive MM6 cells, starting at concentrations higher than 0.05 µg/ml LPS. As can be depicted from Fig. 1(b) (inset), a significantly higher AUC was observed after *E. coli* LPS stimulation as compared with both *P. gingivalis* strains ($p<0.05$). Also, a significantly higher AUC after stimulation with LPS of *P. gingivalis* K^- as compared with *P. gingivalis* K1 was observed ($p<0.05$).

Experiment 2: cytokine production after stimulation of whole blood

No cytokines were detected in plasma from unstimulated blood samples (data not shown). Apart from IL-6 production after *P. gingivalis* K1 stimulation, stimulation with *E. coli* induced an overall higher cytokine production as compared with *P. gingivalis* K^- and *P. gingivalis* K1 stimulation ($p<0.05$) (Fig. 2). Furthermore, stimulation with *P. gingivalis* K^- resulted in a significantly lower overall cytokine production than *P. gingivalis* K1 ($p<0.05$, for all cytokines tested).

Cytokine production was higher after whole blood stimulation with *E. coli* LPS, as compared with the LPS of *P. gingivalis* K^- and *P. gingivalis* K1 ($p<0.05$, for all cytokines tested*)* (Fig. 3). Stimulation with *P. gingivalis* K^- LPS induced a significant higher overall cytokine production than *P. gingivalis* K1 LPS ($p<0.05$, for all cytokines tested).

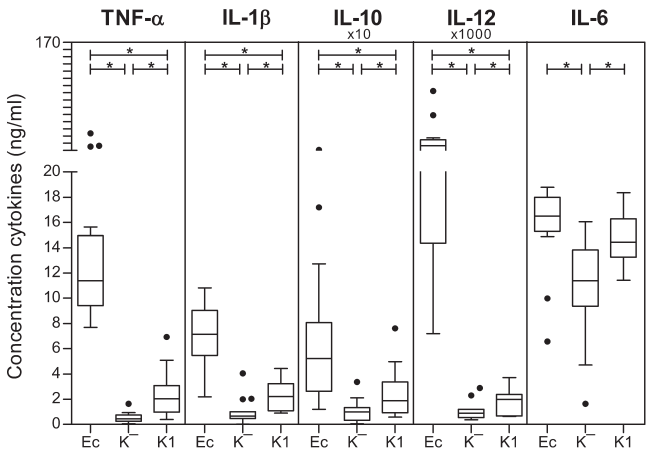

Fig. 2 – Median, 25th and 75th percentiles and 1.5 interquartile range (error bar) of secretion of TNF-α, IL-1β, IL-10, IL-12 and IL-6 in human whole blood following stimulation with $5x10^7$ bacteria/ml of *E. coli* (Ec), *P. gingivalis* K^- (Pg K^-) or *P. gingivalis* K1 (Pg K1). Outlier values are represented as individual points: ●. (*) Significantly different (Wilcoxon's Signed Rank test with Bonferroni's correction; $p<0.05$).

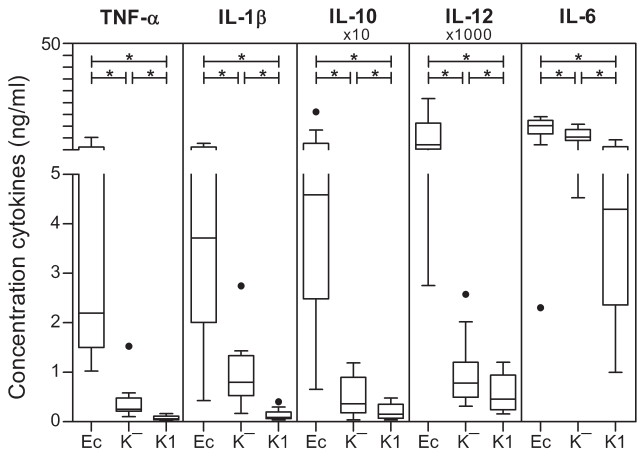

Fig. 3 – Median, 25th and 75th percentiles and 1.5 interquartile range (error bar) of secretion of TNF-α, IL-1β, IL-10, IL-12 and IL-6 in human whole blood following stimulation with 2 µg/ml LPS of *E. coli* (Ec), *P. gingivalis* K^- (Pg K^-) or *P. gingivalis* K1 (Pg K1). Outlier values are represented as individual points: ●. (*) Significantly different (Wilcoxon's Signed Rank test with Bonferroni's correction; $p<0.05$).

Pro-/anti-inflammatory ratios

We observed that cytokine production was induced differently following exposure to the various bacteria or LPS. Therefore, we determined the ratio between pro-inflammatory and anti-inflammatory cytokines after the various stimulations.

The TNF-α/IL-10 ratios after both *P. gingivalis* bacterial stimulations were lower than after *E. coli* stimulation ($p<0.05$) (Fig. 4(a)). There were no differences in the TNF-α/IL-10 ratios between the two *P. gingivalis* bacterial strains. Fig. 4(b) shows the TNF-α/IL-10 ratios after the various LPS stimulations. No differences in the TNF-α/IL-10 ratios were observed between stimulation with LPS of *E. coli* as compared with both *P. gingivalis* strains. However, a higher TNF-α/IL-10 ratio was observed after *P. gingivalis* K^- LPS stimulation as compared with *P. gingivalis* K1 ($p<0.05$).

IL-12 is an important pro-inflammatory cytokine associated with aggressive periodontal disease[25], forming a link between innate and adaptive immunity[26]. Therefore, we calculated the ratios of IL-12/IL-10 after the various stimulations. A significantly higher IL-12/IL-10 ratio was seen after stimulation with *E. coli* bacterial stimulation as compared with both *P. gingivalis* strains ($p<0.05$) (Fig. 5(a)). No significant differences in the IL-12/IL-10 ratios between the two *P. gingivalis* strains were observed after bacterial stimulation. Fig. 5(b) shows the IL-12/IL-10 ratios after the various LPS stimulations. There were no differences in the IL-12/IL-10 ratios between the various stimulations.

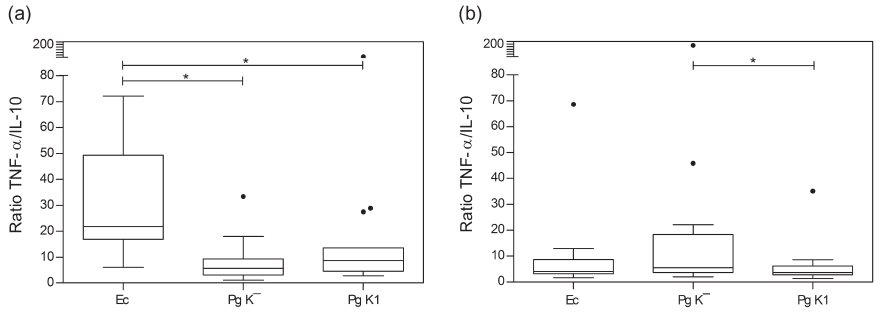

Fig. 4 – Median, 25th and 75th percentiles and 1.5 interquartile range (error bar) of TNF-α/IL-10 ratios: (a) following stimulation with 5x10^7 bacteria/ml of *E. coli* (Ec), *P. gingivalis* K$^-$ (Pg K$^-$) or *P. gingivalis* K1 (Pg K1) and (b) after stimulation with 2 µg/ml LPS of Ec, Pg K$^-$ or Pg K1. Outlier values are represented as individual points: ●. (*) Significantly different (Wilcoxon's Signed Rank test with Bonferroni's correction; $p<0.05$).

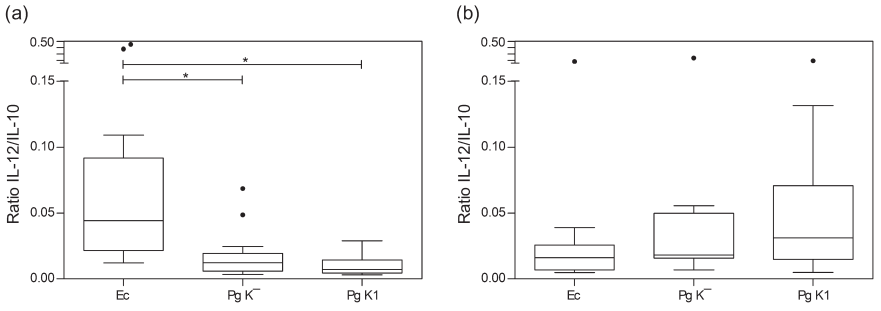

Fig. 5 – Median, 25th and 75th percentiles and 1.5 interquartile range (error bar) of IL-12/IL-10 ratios: (a) following stimulation with 5x10^7 bacteria/ml of *E. coli* (Ec), *P. gingivalis* K$^-$ (Pg K$^-$) or *P. gingivalis* K1 (Pg K1) and (b) after stimulation with 2 µg/ml LPS of Ec, Pg K$^-$ or Pg K1. Outlier values are represented as individual points: ●. (*) Significantly different (Wilcoxon's Signed Rank test with Bonferroni's correction; $p<0.05$).

Discussion

This study was undertaken to investigate whether differences in virulence properties between *P. gingivalis* K$^-$ and *P. gingivalis* K1 may be due to differences in their capacities to induce cytokine production by monocytes. As expected, we showed that bacteria and LPS of both *P. gingivalis* strains were less potent inducers of cytokines as compared with our reference strain *E. coli*. In line with our hypothesis, we found prominent differences in cytokine production following incubation with *P. gingivalis* K$^-$ vs. *P. gingivalis* K1 bacteria or LPS. *P. gingivalis* K$^-$ bacteria in general induced a lower cytokine production as compared with *P. gingivalis* K1. This difference can not be due to differences in

cytokine stimulation by their LPS, since *P. gingivalis* K⁻ LPS induced a higher cytokine production as compared with LPS of *P. gingivalis* K1.

The minor differences in results between experiments 1 and 2 can be explained by the use of different experimental protocols, i.e. use of MM6 vs. whole blood, use of intracellular cytokine production vs. extracellular production, 4 h stimulation vs. 24 h. Despite these minor differences, in both experiments, we found a higher overall cytokine production and higher TNF-α/IL-10 and IL-12/IL-10 ratio after *E. coli* bacterial stimulation as compared with *P. gingivalis* bacterial stimulation. This higher pro-inflammatory cytokine production following *E. coli* stimulation was expected, since it is a well-established fact that *E. coli* is a more potent inducer of pro-inflammatory cytokines than *P. gingivalis* both in vivo[17] and in vitro[16]. The production of IL-6, however, appeared to be much more similar between *E. coli* bacteria and the *P. gingivalis* species. This may have an in vivo relevance, since IL-6 plays an important role in regulating the immune response and leukocyte recruitment[27]. It also stimulates bone resorption by stimulating the formation and activation of osteoclasts[28]. IL-6 may therefore play an important role in the pathogenesis of periodontal diseases.

In addition, the overall decreased production of all cytokines by inflammatory cells after bacterial stimulation with *P. gingivalis* K⁻ as compared with *P. gingivalis* K1 is in line with previous findings[8;9]. The higher total cytokine production may play a role in the more invasive character of the infection following *P. gingivalis* K1 infection in vivo. Increased cytokine production may enhance the spreading of the infection by activating other leukocytes in the environment and by attracking these cells to the site of infection[28]. Various bacterial substances may be responsible for the differences in cytokine induction after *P. gingivalis* K⁻ compared with *P. gingivalis* K1 stimulation. The type of fimbriae of *P. gingivalis* K1 (type IV *fimA*) has been reported to induce a much stronger systemic inflammation in a mouse model as compared with the type of fimbriae of *P. gingivalis* K⁻ (type 1 *fimA*)[29]. Differences in cysteine proteinase structure may also have accounted for the observed variety in cytokine production between the two *P. gingivalis* strains[30]. Furthermore, the presence of capsular polysaccharides in the bacterial suspension of *P. gingivalis* K1 could have contributed to the observed differences between the bacterial strains. The capsule of *P. gingivalis* K1 has been shown to reduce phagocytosis in vivo, providing the bacterium with a mechanism to evade internalisation and clearance by host inflammatory cells[31]. A decreased phagocytosis of these bacteria may result in increased numbers of bacteria which can be recognised by monocytes, leading to increased cytokine production. However, recent studies looking at cytokine production by inflammatory cells after stimulation with encapsulated *P. gingivalis* and non-encapsulated mutants have shown higher cytokine production in the non-encapsulated mutant[31;32]. Additional in vivo studies are thus needed to elucidate the role of the capsule of *P. gingivalis* in host recognition and subsequent

inflammatory responses.

Differences in chemical characterisation and biologic properties of the LPS[33] may also have played a role, since the LPS is considered as one of the most important pro-inflammatory molecules of gram-negative bacteria[2]. Although *E. coli* LPS and *P. gingivalis* LPS differ in chemical structure of the lipid A species[34], and signal through different Toll-like receptors (TLR2 for LPS of *P. gingivalis*[35] and TLR4 for LPS of *E. coli*[36,37]), we found no differences in the IL-12/IL-10 and TNF-α/IL-10 ratio between *P. gingivalis* LPS vs. *E. coli* LPS. Our results are thus not in line with the suggestion that TLR2 is a weak inducer of pro-inflammatory cytokines[38]. It, however, corroborates the suggestion that TLR2 activation may also induce strong type 1 helper T cell (T_H1) responses[39] and indicates that TLR2 activation may induce pro-inflammatory or immunomodulatory signalling[38;40].

From our results, it is unlikely that LPS of the *P. gingivalis* strains is responsible for the observed differences in cytokine production following stimulation with *P. gingivalis* K^- and K1 bacteria. In contrast to *P. gingivalis* bacterial stimulation, we observed a higher overall cytokine production after incubation with *P. gingivalis* K^- LPS versus incubation with *P. gingivalis* K1 LPS and a higher TNF-α/IL-10 ratio. Our results are in agreement with a study of Bramanti et al.[33], who also showed higher TNF-α and IL-1β production after stimulation of inflammatory cells with LPS of *P. gingivalis* K^- as compared with LPS of *P. gingivalis* K1. This may be due to differences in the chemical properties of the LPS between these two *P. gingivalis* strains, since variations in carbohydrate and galactosamine composition and lipid A proportion between the strains have been observed[33]. Therefore, our findings do not support the idea that the LPS of *P. gingivalis* plays an important role in the observed variations in virulence properties between the species[2]. Other bacterial products than LPS seem to be responsible for the higher cytokine production following stimulation with *P. gingivalis* K1 bacteria compared with *P. gingivalis* K^- bacteria.

In summary, although *E. coli* bacteria were more potent inducers of cytokine production in whole blood, we also observed differences in cytokine production after stimulation with *P. gingivalis* K^- vs. *P. gingivalis* K1: stimulation of whole blood with the encapsulated *P. gingivalis* K1 bacteria resulted in an overall higher cytokine production. This may be related to the increased virulence of the encapsulated *P. gingivalis* strain, since a higher cytokine production may result in the attraction of more and other leukocytes and increase the spreading of the inflammatory response. The reason why *P. gingivalis* K1 induced a higher cytokine production remains elusive from the present study, since the most obvious cytokine inducing substance from gram-negative bacteria and thus *P. gingivalis*, its LPS, did not induce a higher cytokine production as compared with LPS from *P. gingivalis* K^-. Therefore, future studies need to be directed towards identifying this mechanism as well as towards revealing a causal relationship

between the higher cytokine production of *P. gingivalis* K1 and its higher virulence properties.

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CHAPTER 7

General discussion

General discussion

The purpose of the present thesis was to evaluate the putative association between periodontitis and preeclampsia. First, we evaluated epidemiological and scientific evidence for such a relationship in the literature and conducted a case-control study, in which we compared the periodontal condition of women with a recent history of preeclampsia and women with uncomplicated pregnancies. Then we studied in the rat whether *P. gingivalis* LPS, as an important antigen in periodontal infections, could induce pregnancy complications, including preeclampsia. We observed that infusion of LPS from *P. gingivalis* into pregnant rats induced hypertension, decreased placental and foetal weight and induced foetal resorptions. The response of pregnant rats to *P. gingivalis* LPS was different from the response of pregnant rats to the well established pro-inflammatory LPS of *E. coli*, which induces a preeclampsia like syndrome, including hypertension, proteinuria and generalised inflammation. This difference in response may be induced by variations in cytokine production. Therefore we incubated whole blood of pregnant women with LPS as well as bacteria of *P. gingivalis* and *E. coli* and measured cytokine production and also compared the response of human leukocytes to a non-virulent and a virulent serotype of *P. gingivalis*.

Epidemiological associations

Adverse pregnancy outcomes including preterm delivery, foetal growth restriction and preeclampsia affect a significant number of pregnancies, often accompanied with both maternal and foetal morbidity and mortality[1-3]. Infections and subsequent host inflammatory processes remain important risk factors for pregnancy complications and continue to be investigated as a potential causative factor for adverse pregnancy outcomes, especially in pregnancies complicated with preeclampsia[4;5]. The possible relationship between periodontitis and preeclampsia has been studied in various populations, with conflicting findings[6-26]. *Chapter 2* provides an overview of the primary studies conducted from 2003 until 2010. Analysis of the data showed that, although findings were not consistent, there are indications that periodontitis may be associated with preeclampsia. A meta-analysis conducted by Ide and Papapanou in 2013 confirmed significantly deeper probing depths and higher levels of gingival bleeding in preeclamptic pregnancies as compared with healthy controls[27]. Some of the conflicting findings observed in *chapter 2* may be due to the fact that the association between periodontitis and preeclampsia was investigated in several ethnically different populations. Ethnicity has been shown to be a risk factor for both periodontitis as well as preeclampsia in the past[28;29] and may therefore have biased study outcomes. Another major problem might have been the variation in defining periodontal disease. Until now there is no accepted 'gold standard' definition for periodontitis. It appears that the strength of the association between periodontitis and adverse pregnancy outcomes

may depend upon the adopted periodontitis definition[30]. When reviewing the available literature, it became apparent that a large variety of methods was adopted to define periodontitis as a risk factor for preeclampsia. It is of interest that all studies that, next to pocket probing depth and/or clinical attachment level also included bleeding on probing (BOP), indicating active inflammation, in their periodontal disease definition found an association between periodontitis and preeclampsia[7;8;11;17;31]. Because we were aware of this, in *chapter 3* we tried to obtain consistency by stratifying periodontitis according to the definitions used by Boggess et al[31] who also included BOP. Indeed, we confirmed a strong association between periodontitis and preeclampsia. In contrast, most studies that did not include BOP in their definition failed to find an association[9;15;18;25]. Therefore, the adopted criteria for defining periodontitis in the observational studies reviewed may not have provided the proper tool to draw a decisive conclusion on periodontitis as a risk factor for preeclampsia, because not always an active inflammatory process is involved. This emphasises the importance of a universal standardised periodontal disease definition that includes the inflammatory burden. Nesse et al therefore recently developed the PISA (Periodontal Infected Surface Area) definition for periodontal disease[32]. This model calculates the extension of the infected periodontal surface in square millimetres, reflecting the severity of the periodontal infection, and quantifies the total inflammatory burden. PISA has recently been shown to be a useful model to determine a dose-response relationship between the inflammatory burden posed by periodontitis and the level of glycated haemoglobin (HbA1c) in type 2 diabetes mellitus patients[33]. In fact, Nesse and coworkers compared several periodontal disease definitions and found that PISA reflected the inflammatory burden better than all other measures of periodontitis severity. Using PISA might be a major step forward in investigating the association between periodontitis and preeclampsia. The consensus report of the Joint European Federation of Periodontology (EFP) and American Academy of Periodontology (AAP) Workshop on Periodontitis and Systemic Diseases: Periodontitis and adverse pregnancy outcomes (2013) now also suggests the use of exposure-response relationship analyses, next to categorical assessments, when investigating the role of periodontitis in pregnancy complications[34].

Another point of consideration may be the fact that there are several classifications for preeclampsia in the literature: severe and mild preeclampsia (with a consensus threshold for severe preeclampsia, being systolic blood pressure of >160 mmHg and a diastolic blood pressure of >110 mmHg with proteinuria)[35] and early-onset (<34 weeks of gestation) and late-onset (>34 weeks of gestation) preeclampsia[36;37]. Early-onset en late-onset preeclampsia are thought to be two different syndromes, for they differ in pathogenesis, genetic risk and inheritance[37;38]. Early-onset preeclampsia starts with abnormal placentation (placental preeclampsia) and is associated with reduced placental volume, low birth weight, perinatal death and adverse maternal outcome[39].

This abnormal placentation and foetal growth restriction is usually not seen in late-onset preeclampsia (maternal preeclampsia). Late preeclampsia is considered to arise from the interaction between a normal placenta and maternal factors that predispose to microvascular diseases, such as diabetes mellitus and long-term hypertension[36]. In these metabotically or genetically predisposed women, the demands of the growing foeto-placental unit at the end of pregnancy exceeds the maternal resources[40]. Since in *chapter 2* and in our case-control study described in *chapter 3* it was shown that a remarkable high incidence of periodontitis was observed in early-onset preeclampsia, this could indicate that an association between the two diseases may only be evident in early-onset preeclampsia. Most of the studies included in the systematic review described in *chapter 2*, did not discriminate between early-onset and late-onset preeclampsia, which may have biased study outcomes. Therefore, like standardised periodontal disease definitions, also universal standardised preeclampsia definitions should be adopted in order to elucidate a relationship between the two diseases.

It can be concluded that periodontitis and preeclampsia may be associated depending on the inflammatory state of periodontitis and especially in women with early-onset preeclampsia. However, it is unclear whether periodontitis plays a causal role in the pathogenesis of preeclampsia, because studies focusing on the periodontal condition before 20 weeks of gestation, i.e. before the occurrence of the clinical symptoms of preeclampsia, failed to show an association[25;31]. Also in our case-control study (*chapter 3*), in which periodontitis was recorded 3-28 months after delivery, it can not be excluded that the preeclamptic state may have induced or aggrevated periodontitis rather than vice versa. However, none of these studies were designed to demonstrate that preeclampsia may have been a risk factor for the induction or progression of periodontitis. Therefore, from the observational studies described in *chapter 2*, it remains unclear whether the relationship between the two diseases is causal or just associative.

Efficacy of periodontal therapy on reducing preeclampsia during pregnancy

The question whether periodontitis plays a causal role in preeclampsia is reinforced by the observation that there appeared to be no beneficial effect of periodontal treatment at ±20 weeks of gestation on the risk of developing preeclampsia (*chapter 2*). However, it is of notice that although the periodontal intervention (primary mechanical therapy, i.e. scaling and root planing and improving oral hygiene) resulted in significant clinical improvement, in none of the treatment studies reviewed in *chapter 2*, the intervention completely restored periodontal health (defined as shallow probing depths of ≤3 mm in the absence of BOP). In fact, Offenbacher et al reported a further increase in incidence or progression in probing depth between baseline and delivery in 40% of the treatment group[41]. These findings suggest that primary mechanical therapy alone may not be

adequate to control gingival inflammation during pregnancy. Success of mechanical therapy is largely dependent on whether specific pathogenic micro-organisms are (still) present at subgingival sites at the completion of the therapy[42]. Especially *P. gingivalis* has been shown to have the capacity to invade into the periodontal tissues[43] and can be difficult to remove by scaling and root planing alone. Indeed, *P. gingivalis* has been associated with refractory and progressive periodontitis and is commonly found in sites that exhibit recurrence and progression[44;45]. Remarkingly, although observational studies showed a higher prevalence of *P. gingivalis*, *E. corrodens, T. forsythia*[11;46] and *P. micra*[17] (*chapter 3*) in the subgingival plaque of preeclamptic women, none of the treatment studies reviewed in *chapter 2* investigated the bacterial composition of the subgingival microbiome before or after treatment. Therefore, in the treatment studies, residual existence of pathogens in the subgingival area, such as *P. gingivalis,* may have resulted in a persistent inflammatory process. A beneficial effect of periodontal therapy on pregnancy outcome is not to be expected if the intervention itself was not successful in returning the women to a healthy periodontal status, for it may not have adequately reduced the level of systemic burden. This may be an explanation for the observation that the treatment studies failed to reduce the risk on preeclampsia and emphasises the importance of determining the bacterial composition of women suffering from periodontitis during pregnancy. Thus, when periodontal pathogens like *P. gingivalis* are found in the subgingival plaque of pregnant women, adjunctive treatment, such as the use of antibiotics and/or periodontal surgery, may be necessary to successfully reduce the systemic burden posed by periodontitis. It is also possible that periodontal therapy at ±20 weeks of gestation was too late in pregnancy to prevent complications, for translocation of micro-organisms such as *P. gingivalis*, or bacterial products like LPS from the periodontal infection into the maternal circulation may already have occurred before therapy[47;48]. This may be of importance, since haematogenous transport of bacteria and/or pro-inflammatory mediators like LPS of gram-negative pathogens from sites of the periodontal infection into the maternal circulation and/or foeto-placental tissues may have provoked a systemic or local inflammatory response, thereby inducing pathological processes that led to these adverse outcomes[49;50].

Biological mechanisms

Since especially *P. gingivalis* can disseminate into the maternal tissue and some studies found a higher prevalence of *P. gingivalis* in subgingival plaque samples of preeclamptic women as compared with healthy pregnant controls[11;46], we studied the potential causal role of *P. gingivalis* in preeclampsia. To do so, we used pregnant rats, which were infused with LPS of *P. gingivalis*, the most prominent pro-inflammatory factor of this bacterium (*chapter 4*). We used the set up of a well established model for preeclampsia, in which pregnant rats were infused on gestational day 14 with a low dose of LPS of the enteric

micro-organism *E. coli*[51]. A rat model was chosen, because rats, just like humans, have a haemochorial placenta and deep trophoblast invasion in the mesometrial triangle[52;53]. We investigated whether LPS of *P. gingivalis* could induce hypertension and albuminuria, could decrease placental or foetal weight or could increase foetal resorptions. Moreover, since *P. gingivalis* LPS is highly pro-inflammatory[54] and may provoke local and systemic inflammatory responses leading to pregnancy complications, including preeclampsia[49], we also examined signs of peripheral inflammation, by measuring total leukocyte counts and differential leukocyte counts as well as signs of placental and glomerular inflammation. From our findings it was shown that infusion with LPS of *P. gingivalis* in pregnant rats increased maternal systolic blood pressure, induced placental and foetal growth restriction and increased the number of foetal resorptions. In contrast with the findings of Faas et al who used *E. coli* LPS[51;55;56], we did not observe albuminuria. We did also not find signs of inflammation in the peripheral blood, in the placenta or in the kidney of pregnant rats after infusion of *P. gingivalis* LPS.

Because placental weight was negatively affected by *P. gingivalis* LPS, we hypothesise that the observed foetal and maternal pathology may have been of placental origin. Recently, it has been found that *P. gingivalis* bacteria can induce G_1 arrest and increase apoptosis of extravillous trophoblast cells[57]. Extravillous trophoblast cells are not only essential for the physical adherence of the placenta to the uterine wall, but also invade and remodel the maternal uterine spiral arteries in the placental bed (the mesometrial triangle in the rat), which is crucial for normal growth and development of the foetus[58]. An increase in extravillous trophoblast apoptosis may lead to an impaired invasion pattern and impaired spiral artery remodeling[52], to a small placenta and subsequently to impaired placental perfusion[59]. Impaired placental perfusion may result in reduced oxygen and nutrients to the foetus[60] and in some foetuses to growth restriction or demise. Because *P. gingivalis* LPS is a TLR2 ligand[61] and activation of TLR2 on extravillous trophoblast cells may induce apoptosis of these cells[62], *P. gingivalis* LPS may have had a direct effect on trophoblast cells and induced increased trophoblast apoptosis. This may have resulted in decreased trophoblast invasion and placental and foetal pathology. This hypothesis needs to be tested in future studies. It also needs to be elucidated what mechanism caused the modest rise in maternal blood pressure in pregnant rats. Although *P. gingivalis* LPS might have had a direct effect on the maternal endothelium[63], it is also possible that the increased blood pressure was a compensatory attempt of the mother to maintain normal blood flow to the foetus in the face of reduced placental perfusion. Such a mechanism has also been proposed to occur in humans[64].

The lack of dose dependency for effects of *P. gingivalis* LPS on foetal resorptions, foetal weight and maternal blood pressure in this model is interesting. Such a lack of dose dependency is in contrast with infusion of *E. coli* LPS, in which a dose dependent effect on foetal resorptions and foetal weight was observed[51]. The reason for lack of dose

dependency for *P. gingivalis* LPS is unclear. It is most likely not due to a lack of a dose dependent increase in cytokine production following *P. gingivalis* LPS infusion, since in *chapter 6* we showed a dose dependent increase in cytokine production following stimulation of monocytes with *P. gingivalis* LPS. However, in *chapter 5* we observed that *P. gingivalis* LPS induced a more anti-inflammatory cytokine ratio as compared with *E. coli* LPS in blood of pregnant women. If a similar anti-inflammatory cytokine pattern is observed in pregnant rats following *P. gingivalis* LPS infusion, the anti-inflammatory cytokines may have prevented LPS-induced foetal growth restriction and foetal loss in these animals[65]. Furthermore, in *chapter 4*, we infused *P. gingivalis* LPS with a single dose at gestational day 14. Although this is relatively late in pregnancy, we still found obvious effects. We chose gestational day 14, because in rat pregnancy, around that day the placenta is fully developed and will only grow in the last week, when trophoblast invasion into the mesometrial triangle begins[52;53]. As indicated before, this trophoblast invasion is necessary to maintain adequate placental perfusion in the last week of pregnancy. This allowed us to study the effect of the LPS on placental and foetal growth rather than on placental and foetal development. However, in humans, pregnant women with periodontitis may be exposed to *P. gingivalis* and/or LPS much earlier in pregnancy, most likely from the beginning of pregnancy. Therefore, for future studies, it would be interesting to infuse LPS at an earlier stage of rat pregnancy, for instance at day seven, three days after implantation of the blastocyst. A more severe response to the LPS could then be expected, since LPS, which may directly affect the trophoblast via TLR2, may also affect placental development. Moreover, pregnant women with periodontitis may be exposed to LPS more continuously. Such a permanent exposure to LPS may also result in more severe complications, for continuous LPS exposure may induce continuous apoptosis of trophoblasts. It is therefore also of interest to investigate the effects of chronic exposure to *P. gingivalis* LPS in pregnant animals, for instance by means of a mini osmotic pump which allows for a permanent systemic release of LPS into the circulation.

It is also of notice that, in *chapter 4*, increasing the dose of *P. gingivalis* LPS did not induce more severe maternal effects in pregnant rats than the lower dose of *P. gingivalis* LPS, again in contrast with *E. coli* LPS[51]. This may suggest that the sensitivity of pregnant individuals to different bacteria or their LPS differs. Such a variety in sensitivity to different LPS species may arise from different induction of cytokine production following contact of the LPS with leukocytes. We therefore evaluated whether the diverse effects of *E. coli* LPS and *P. gingivalis* LPS in the pregnant rat may be due to differences in cytokine production upon stimulation with LPS. For this purpose, blood was taken from pregnant individuals and was stimulated with LPS of *P. gingivalis* and LPS of *E. coli*. This is described in *chapter 5*. For comparison, we also included blood of non-pregnant women, while we also stimulated blood of pregnant and non-pregnant

with whole bacteria of *P. gingivalis* and *E. coli*. In general, we found that *P. gingivalis* (both the whole bacteria and the LPS) was a less potent inducer of both pro- and inflammatory cytokines than *E. coli*, in both blood of pregnant and non-pregnant women. Also, a generally lower pro-inflammatory cytokine ratio was observed following stimulation with bacteria/LPS of *P. gingivalis* as compared with *E. coli*. We hypothesise that this lower cytokine production and lower pro-inflammatory cytokine ratio following *P. gingivalis* LPS vs. *E. coli* LPS stimulation may be involved in the in vivo differences observed in *chapter 4*, in which infusion with LPS of *P. gingivalis* did not induce systemic inflammation and subsequent preeclamptic symptoms in pregnant rats. However, not all cytokine levels were equally affected by *P. gingivalis* or its LPS during pregnancy. For example, the production of IL-6 was reduced during pregnancy as compared with the non-pregnant situation after *P. gingivalis* stimulation. IL-6 is a multifunctional cytokine with both pro- and anti-inflammatory capacities[66]. This cytokine has an important role in the cytokine network that directs the shift from acute to chronic inflammation[67] and is associated with several chronic inflammatory diseases[68], including periodontitis[69]. IL-6 may also be a candidate player in several pregnancy complications, such as preterm delivery, miscarriage and also preeclampsia in both animals and humans[70]. The reason why especially the production of IL-6 upon *P. gingivalis* stimulation is decreased during pregnancy is unclear. Further studies will be necessary to elucidate the mechanisms responsible for the lower IL-6 production upon *P. gingivalis* stimulation during pregnancy, and whether this response is beneficial or detrimental to pregnancy.

In *chapter 4*, we observed the well known pregnancy-induced increased sensitivity to LPS[51;55;71], for the effects of *P. gingivalis* LPS on blood pressure were only observed in pregnant animals. This may be due to different cytokine responses upon LPS treatment of pregnant animals as compared with non-pregnant animals. Therefore, in *chapter 5*, we compared cytokine production after stimulation of blood of pregnant women with cytokine production after stimulation of blood of non-pregnant women. In line with our expectation, pregnancy itself had an effect on cytokine production: the production of cytokines as well as the pro-inflammatory ratios of these cytokines after *E. coli* and *P. gingivalis* bacteria/LPS stimulation in general were comparable or lower in pregnant women than in non-pregnant women. Apparently, monocytes of pregnant women produce less cytokines upon bacterial/LPS stimulation than monocytes of non-pregnant women. Our findings are in line with previous findings of our laboratory, in which it was also observed that the percentages of IL-12 and TNF-α producing monocytes were lower upon LPS stimulation in pregnant women as compared with non-pregnant women[72]. Reduced cytokine production may be the consequence of an endotoxin tolerant state of activated monocytes, caused by factors in the plasma of pregnant women[72] and suggests that pregnant women may be more sensitive to the effects of cytokines. This is in line with earlier results from our lab[73]. However, the exact

biological implication of this pregnancy-induced phenomenon remains to be further investigated.

Finally, in *chapter 6*, we studied the cytokine response upon the different *P. gingivalis* phenotypes and their LPS, since it has been shown that the bacterial phenotype may influence inflammatory responses against these bacteria, even within the same bacterial species. It has been reported that there may be differences in pro-inflammatory properties between LPS of different *P. gingivalis* serotypes[74], and several *P. gingivalis* serotypes with paradoxically opposing (stimulatory versus suppressive) effects on host inflammatory responses do exist[75]. Therefore, our final aim was to further study the host response to *P. gingivalis* and its LPS on human cells. For this purpose, we investigated cytokine production in human whole blood following stimulation with bacteria or isolated LPS of two *P. gingivalis* serotypes [non-encapsulated *P. gingivalis* ATCC 33277 (K^-) and encapsulated *P. gingivalis* W50 (K1)] (*chapter 6*). These specific serotypes were chosen, because encapsulated *P. gingivalis* subtypes have been shown to be more virulent in experimental infections than non-encapsulated *P. gingivalis* subtypes[76]. Differences in virulence properties may result from different cytokine production following recognition of the encapsulated or non-encapsulated bacteria by the host immune system[77;78]. As described in *chapter 6*, both bacteria/LPS of both *P. gingivalis* serotypes were significantly less potent inducers of inflammatory cytokines than *E. coli* LPS. When comparing the two *P. gingivalis* serotypes, the LPS of *P. gingivalis* K^- induced a higher cytokine production and a higher pro-/anti-inflammatory ratio than *P. gingivalis* K1, which is in line with previous findings[74]. It confirms that in our rat experiment (*chapter 4*), we used to the appropriate LPS to investigate the effects of this molecule on inflammatory processes during pregnancy. However, encapsulated *P. gingivalis* K1 bacteria induced higher cytokine production than the non-encapsulated *P. gingivalis* K^-. Similar to the findings described in *chapter 5*, this was especially apparent in the production of IL-6. In fact, the production of IL-6 after *P. gingivalis* K1 bacterial stimulation was comparable with *E. coli* bacterial stimulation, which substantiates the general thought that *P. gingivalis* serotypes, by inducing IL-6, may play an important role in the pathogenesis of periodontitis. Interestingly, the LPS of both serotypes did not significantly contribute to the inflammatory capacity of *P. gingivalis*, when tested in non-pregnant individuals. Apparently, other pro-inflammatory factors, such as fimbriae, lipoproteins or capsular components may (also) be involved in the inflammatory capacity of *P. gingivalis*[54]. This may implicate that in future experimental studies, not isolated LPS alone but rather whole bacteria should be infused to further investigate the role of *P. gingivalis* in pregnancy complications.

The overall higher cytokine production following stimulation with *P. gingivalis* K1 vs. *P. gingivalis* K^- might be related to the higher virulence properties of *P. gingivalis* K1, for increased cytokine production may enhance the spreading of the inflammatory

response. The higher inflammatory potential of *P. gingivalis* K1 may therefore play a role in the more invasive character of the infection following stimulation in vivo. This higher invasive capacity and observed higher inflammatory potential of *P. gingivalis* K1 might be highly relevant during pregnancy, for it may provide this serotype with a mechanism to disseminate into placental tissues more easily than non-encapsulated *P. gingivalis* serotypes. This implicates that not all *P. gingivalis* serotypes may be (equally) involved in pregnancy complications and emphasises the importance of using well defined bacterial subtypes when investigating the effects of *P. gingivalis* on pregnancy outcome. If such experimental studies would show that indeed not all *P. gingivalis* serotypes equally affect pregnancy outcome, then identifying the specific *P. gingivalis* serotypes in the subgingival plaque of pregnant women might become a next step in investigating a causal role of periodontitis in pregnancy complications.

Conclusions, future perspectives and clinical implications

This thesis showed that periodontitis may be associated with pregnancy complications, such as preeclampsia, and also provides some new insights into the identification of a mechanism by which the periodontal infection may be involved in adverse pregnancy outcomes. Observational studies indicate that periodontitis may be associated with some forms of preeclampsia. This was most obvious in early-onset preeclampsia (*chapter 2* and *chapter 3*). It is however questionable whether periodontitis is causally involved in preeclampsia, because this thesis not only showed that studies focusing on the periodontal condition before 20 weeks of gestation, i.e. before the clinical occurrence of preeclampsia, failed to find an association, also periodontal treatment performed at about 20 weeks of pregnancy did not reduce the risk on preeclampsia (*chapter 2*). Moreover, one of the most virulent antigens of the periodontal infection, *P. gingivalis* LPS, did not induce preeclamptic features or systemic inflammation in the pregnant rat (*chapter 4*), nor induced strong pro-inflammatory cytokine production in our in vitro and ex vivo experiments (*chapter 5* and *chapter 6*). It therefore remains unclear whether the preeclamptic state predisposes to periodontitis or vice versa or that a confounding factor predisposes to both diseases.

The rat experiment, however, demonstrated that the LPS of *P. gingivalis* may induce some pregnancy complications, such as hypertension, foetal growth restriction, foetal death or miscarriage. It is possible that LPS of *P. gingivalis* is involved in trophoblast apoptosis, although the exact mechanism by which this molecule is involved in obstetric complications remains to be established. It is widely appreciated that aberrations of the normal trophoblast invasion pattern into spiral arteries may lead to pregnancy complications in humans. Therefore, more comprehensive research to pathological mechanisms, and especially experimental research to the role of specific pathogenic micro-organisms in placental bed pathology, should be performed.

Our findings indicate that haematogenous spread of *P. gingivalis* LPS in rats affects placental growth, which may be one of the causes of adverse obstetric outcomes. It needs to be established whether *P. gingivalis* or its LPS also affects placental growth in humans. It also needs to be established whether the association between *P. gingivalis* and adverse pregnancy outcomes may depend on the specific serotype present. Early detection and therapeutic elimination of *P. gingivalis* might then become important intervention strategies. In this thesis we chose to focus on *P. gingivalis*, because this micro-organism is one of the most important pathogens associated with severe periodontitis[79], expresses appropriate virulence properties in animal studies[80] and has been associated with foetal growth restriction and foetal resorptions in animal experiments in the past[80-82]. However, it is possible that other bacterial species from the periodontal infection, such as *A. actinomycetemcomitans*, *T. forsythia*, *P. intermedia*, *T. denticola* and *F. nucleatum*, alone or in synergy, may also be involved in pregnancy

complications, such as preeclampsia[83;84]. This should be investigated in future studies.

Clearly, at this moment it is unknown whether periodontal therapy in early pregnancy or before conception will effectively reduce the risk on developing pregnancy complications. Therefore, there is great need for a large randomised study of preconception or interception screening and treatment of periodontitis. Such a study may also identify which patients would benefit from screening and treatment and which screening methods would be useful. Until then, the 'Guidelines for Treating the Pregnant Patient' of the American Academy of Periodontology[85] remain applicable. In this statement it is recommended that preventive oral care should be performed as early as possible in pregnancy and if needed, scaling and root planing should be scheduled early in the second trimester. Preventive oral care during pregnancy should not only include training and motivation in oral hygiene practices, but also include education in periodontal self-assessment, so that the pregnant patient may recognise the obvious signs of gingival inflammation and seek the appropriate oral care, if necessary[34]. Irrespective of an aetiological role of periodontitis in pregnancy complications, the altered maternal immuno-responsiveness during pregnancy and the presence of *P. gingivalis* increases the susceptibility to gingival inflammation during pregnancy[86;87]. Since oral health is an integral part of general health, early detection and treatment of periodontal diseases remain unquestionably of benefit to the mother. Especially in the Netherlands, were both obstetric and oral care are very well organised, and periodontal screening is part of the routine annual dental check-up, it should be possible to screen and treat women in the reproductive age for periodontitis and follow them throughout gestation. Such a strategy clearly has the advantage of reducing the need for periodontal intervention during pregnancy, when women are at increased risk on developing gingival inflammation and/or worsening of the periodontal condition. In order to achieve this, not only a closer collaboration between professionals in obstetric care and oral care is necessary, but also public health officials should emphasise on the importance of preconceptional and interceptional periodontal screening and/or treatment.

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CHAPTER 8

Summary

Summary

The general introduction *(chapter 1)* briefly describes the pathophysiology of periodontal diseases, the possible systemic consequences of the periodontal infection and also its putative role in pregnancy complications, with a special focus on preeclampsia.

It was first suggested in 2003 that periodontitis during pregnancy might be related with preeclampsia, a severe complication of pregnancy clinically manifest by hypertension and proteinuria during the second half of pregnancy. There is growing evidence that a chronic generalised inflammatory response is an important factor in the pathogenesis of preeclampsia. Periodontitis, as a chronic infection, might superimpose on the already pro-inflammatory state of normal pregnancy, thereby inducing preeclampsia. Periodontopathic bacteria or bacterial products from the oral cavity may also enter placental or foetal tissues via the maternal blood stream and induce inflammation at site.

Clearly, at the start of this thesis, there was little evidence to substantiate a relationship between periodontitis and preeclampsia. Therefore, the overall aim of this thesis was to investigate the nature of this putative relationship, and more specifically to explore epidemiological associations between periodontitis and preeclampsia, to investigate the effect of periodontal therapy on preeclamptic rates and also to explore pathological pathways that might explain such a relationship.

The review presented in *chapter 2* aimed to systematically review the current available scientific evidence for an association between periodontal disease and preeclampsia. A search of the literature was performed in the computerised databases of MEDLINE via PubMed (1969-August 2010), EMBASE (1974-August 2010) and CINAHL (2003-August 2010). Relevant studied performed in humans, published in English, German, French, Spanish and Dutch were included. Methodological quality was assessed by two reviewers independently using quality-assessment forms designed by the Dutch Cochrane Collaboration. Out of the primary eligible articles, 12 observational studies and three randomised-controlled trials (RCTs) fulfilled the inclusion and exclusion criteria. Eight of the observational studies reported a positive association, while four studies found no association. The most obvious association was observed between periodontitis and early-onset preeclampsia. However, it appeared difficult to compare the studies, due to variations in adopted definitions of periodontal disease and preeclampsia, timing of periodontal examination and inadequate control for confounders, such as ethnicity. Since studies focusing on the periodontal condition at enrolment, i.e. before preeclampsia had occurred, failed to show an association, it is unclear whether periodontal disease plays a causal role in preeclampsia. None of the RCTs reported reductions in preeclamptic rates after periodontal treatment performed at about 20 weeks of gestation. The meta-analysis showed a periodontal disease

associated RR on preeclampsia of 1.0 (95% CI 0.78-1.28). It can therefore be concluded that periodontal therapy performed at about 20 weeks of gestation has no beneficial effect on pregnancy outcome.

Since variety in periodontitis and preeclampsia definitions as well as ethnicity may have confounded results in *chapter 2*, in our case-control study described in *chapter 3*, the periodontal condition and subgingival plaque composition of 17 Causasian women with a recent history of early-onset preeclampsia was compared with the periodontal condition of 35 Caucasian women with uncomplicated pregnancies. In order to obtain consistency in periodontal disease definitions, we stratified periodontitis as described by Boggess et al (2003). Full mouth periodontal examinations were performed to determine the periodontal condition and subgingival plaque samples were analysed for the presence of seven of the most important periodontopathic micro-organisms. Severe periodontitis was present in 82% of the preeclamptic women and in 37% of the controls. We found that, after adjusting for age, BMI, smoking and educational level, the periodontal condition of preeclamptic women was worse as compared to women with uncomplicated pregnancies and that severe periodontitis was associated with preeclampsia (adjusted OR 7.9, 95% CI 1.9-32.8). The periodontopathic micro-organism *M. micros* (now referred to as *P. micra*) was more prevalent in the case group while *C. rectus* was more prevalent in the control group. The results indicated that the periodontal condition in Caucasian women with a recent history of early-onset preeclampsia was significantly worse as compared with the periodontal condition of women with uncomplicated pregnancies.

In order to investigate a causal role of periodontitis in pregnancy complications, in *chapter 4* we performed an experimental animal study in which we studied the impact of *P. gingivalis* LPS infusion into pregnant rats. We investigated whether the most virulent component of *P. gingivalis*, its lipopolysaccharide, like LPS of *E. coli*, could induce pregnancy complications, including preeclamptic features, in the rat. For this purpose we conducted two experiments. In experiment 1, on gestational day 14, pregnant rats received increasing doses of *P. gingivalis LPS* (0.0-50.0 µg/kg bw LPS; n=2/3 for each dose). The following parameters were documented: maternal intra-aortic blood pressure, urinary albumin excretion, placental and foetal weight and number of foetal resorptions. In experiment 2, pregnant and non-pregnant rats were infused with 10.0 µg/kg bw *P. gingivalis LPS* (which induced the highest blood pressure and reduced foetal weight in experiment 1), or saline alone (n=7/9 for each group). The same parameters of experiment 1 as well as numbers of peripheral leukocytes and signs of inflammation in the kidney and placenta were evaluated. The results of experiment 1 showed that *P. gingivalis LPS* infusion in pregnant rats increased maternal systolic blood pressure as

well as number of foetal resorptions and reduced placental weight at most doses tested. We did not observe a reduction in foetal weight nor did the rats develop albuminuria. These findings were confirmed in experiment 2 after infusion with 10.0 µg/kg bw *P. gingivalis LPS* (the dose that induced the highest blood pressure together with decreased foetal weight in experiment 1). However, after analysing the number of foetuses that were more than two standard deviations (2 SD) from the normal mean (4.75 ± 0.50 g; i.e. less than 3.75 g), it was shown that a significant higher number of foetuses were growth restricted in the *P. gingivalis LPS* infused group as compared with the saline controls. No signs of a generalised inflammatory response were observed, nor did we find indications for inflammation in the kidney or placenta. The increased blood pressure appeared to be specific for pregnant rats, since no effects of *P. gingivalis LPS* were seen in non-pregnant rats. It can be concluded that *P. gingivalis LPS* affects pregnancy by increasing maternal blood pressure, inducing placental and foetal growth restriction and by increasing the number of foetal resorptions. Therefore, *P. gingivalis LPS* may play a role in pregnancy complications induced by periodontitis. Unlike LPS of *E. coli*, *P. gingivalis LPS* does not appear to play a role in the pathogenesis of the maternal syndrome of preeclampsia, for we did not observe albuminuria nor inflammation.

Therefore, in *chapter 5*, we evaluated whether the observed different effects of *P. gingivalis* LPS and *E. coli* LPS on pregnancy may be due to differences in cytokine production upon recognition by leukocytes. For this purpose, blood samples were taken from 16 healthy pregnant and 15 healthy non-pregnant women and stimulated with bacteria and LPS of *P. gingivalis* ATCC 33277 and *E. coli* in an ex vivo laboratory study. After the stimulations, we measured cytokine production (TNF-α, IL-1β, IL-6, IL-12 and IL-10) using Luminex and TLR2/TLR4 expression on monocytes using flow cytometry. We also calculated pro-/anti-inflammatory ratios (IL-12/IL-10, TNF-α/IL-10 and IL-6/IL-10). The results showed that in both pregnant and non-pregnant women, stimulation with *P. gingivalis* bacteria/LPS induced lower cytokine production and lower pro-inflammatory ratios than *E. coli* bacteria/LPS, with the exception of the IL-6/IL-10 ratio, which was higher after *P. gingivalis* bacteria/LPS stimulation. There was also an effect of pregnancy on cytokine production: the concentration of IL-12 after *E. coli* bacterial stimulation and the concentration of IL-6 after *P. gingivalis* bacterial stimulation was lower in whole blood of pregnant women than in non-pregnant women. Similarly, as compared with non-pregnant women, in pregnant women the production of IL-12 and TNF-α was lower after stimulation with LPS of *E. coli*, and the production of IL-6 was lower after stimulation with *P. gingivalis* LPS. The percentage of TLR2 positive monocytes was lower in pregnant women vs. non-pregnant women. The generally lower cytokine production and lower pro-inflammatory ratio after *P. gingivalis* stimulation vs. *E. coli* stimulation during pregnancy may explain differences in response

upon bacterial recognition in the in vivo situation and suggest that pregnant women are more sensitive to the effects of cytokines like IL-12, IL-6 and TNF-α.

Strain dependent variations in virulence of *P. gingivalis* have been reported. This may be due to differences in cytokine production following recognition of the bacteria or their LPS by host inflammatory cells. Therefore, in order to further investigate virulence factors of *P. gingivalis*, in *chapter 6*, we compared cytokine production following stimulation with bacteria or LPS of two different *P. gingivalis* serotypes. We stimulated the cell-line Mono Mac 6 (MM6) as well as human whole blood from 15 healthy women with bacteria and isolated LPS of the non-encapsulated *P. gingivalis* ATCC 33277 (K^-) and the encapsulated *P. gingivalis* W50 (K1). In experiment 1, TNF-α production following stimulation of the cell-line Mono Mac 6 with bacteria or LPS of both *P. gingivalis* strains was determined using flow cytometry. In experiment 2, we investigated the effects of the two *P. gingivalis* serotypes or their LPS on TNF-α, IL-1β, IL-6, IL-12 and IL-10 production in whole blood collected from 15 healthy women using Luminex. In both experiments, *E. coli* bacteria and LPS were used as a reference. Both *P. gingivalis* serotypes induced lower cytokine production than *E. coli*, with the exception of IL-6 after *P. gingivalis* K1 bacterial stimulation. *P. gingivalis* K1 bacteria elicited a higher overall cytokine production than *P. gingivalis* K^-. In contrast, *P. gingivalis* K1 LPS stimulation induced a lower cytokine production than *P. gingivalis* K^- LPS. Apparently, other bacterial products than LPS seem to be responsible for the higher cytokine production following stimulation with *P. gingivalis* K1 bacteria as compared with *P. gingivalis* K^- bacteria. This stronger cytokine production after stimulation with *P. gingivalis* K1 may contribute to its virulence properties because increased production of cytokines may recruit more leukocytes to the site of infection, thereby enhancing the local inflammatory process.

Finally, in *chapter 7* the main research outcomes are discussed and perspectives for future research are described. From observational studies, including the case-control study conducted by our own group, there appears to be an association between periodontitis and some forms of preeclampsia, most evident early-onset preeclampsia. It is, however, questionable whether periodontitis is causally involved in preeclampsia, because studies that performed periodontal screening before 20 weeks of gestation (i.e. before the occurrence of the clinical symptoms of preeclampsia) failed to show an association. Also, periodontal therapy performed at about 20 weeks of gestation did not reduce the risk on preeclampsia. Therefore, it remains elusive whether periodontitis may induce preeclampsia or vice versa or that an unknown factor predisposes to both diseases. It can, however, not be excluded that periodontal therapy performed at about 20 weeks of gestation may have been too late in pregnancy, for translocation of oral bacteria, such as *P. gingivalis*, into the maternal circulation and foetoplacental tissues

may already have occurred before therapy.

The results of our animal study substantiated previous findings that LPS of *P. gingivalis* may be causally involved in pregnancy complications such as low birth weight and miscarriage, in this thesis presented as foetal resorptions in the rat. Although we observed a slight increase in maternal blood pressure, infusion with *P. gingivalis* LPS did not induce albuminuria or generalised inflammation. LPS of *P. gingivalis* therefore appears not be involved in the pathogenesis of preeclampsia. It can be hypothesised that the foetal pathology and increased blood pressure arose from the placenta as placental weight was decreased by *P. gingivalis* LPS infusion. *P. gingivalis* LPS may have directly affected placental growth by increasing trophoblast apoptosis. This in turn may have led to reduced oxygen and nutrient supply to the foetus and subsequently to growth retardation of the foetus. The slightly increased maternal blood pressure may have been a compensatory mechanism to restore oxygen and nutrient supply to the foetus and as such also be of placental origin. The exact mechanism by which LPS of *P. gingivalis* increased maternal blood pressure and reduced placental and foetal growth remains obscure from our experiment and needs further investigation.

Our in vitro and ex vivo blood experiments showed that both bacteria and LPS of *P. gingivalis* serotypes had lower pro-inflammatory capacity and induced lower cytokine production by monocytes than *E. coli* bacteria and LPS. This may explain why infusion with *P. gingivalis* LPS, in contrast with *E. coli* LPS did not induce preeclamptic features in our animal experiment. Interestingly, despite the fact that pregnant women are extremely sensitive to LPS, cytokine levels in blood of pregnant women after the various stimulations was similar or lower as compared with non-pregnant women. This may reflect an endotoxin tolerant state of activated monocytes during pregnancy and suggests that pregnant women may be more sensitive to the effects of cytokines. Further studies will be necessary to elucidate what mechanisms are responsible for the lower cytokine production upon LPS stimulation during pregnancy.

In conclusion, this thesis shows there are indications from observational studies that human periodontitis may be associated with some forms of preeclampsia. However, no beneficial effect of periodontal therapy on preeclamptic rate was observed, nor did *P. gingivalis* LPS induce preeclampsia or generalised systemic inflammation in the pregnant rat. Also, *P. gingivalis* bacteria or LPS did not induce a strong pro-inflammatory cytokine production in the in vitro en ex vivo blood experiments. It seems therefore unlikely that periodontitis plays a causal role in the pathogenesis of preeclampsia.

Periodontitis may be involved in low birth weight and miscarriage by the presence of *P. gingivalis* LPS, as was shown in a rat experiment. It is of importance to elucidate the exact pathway by which *P. gingivalis LPS* interferes with pregnancy. Interestingly, in contrast with the common hypothesis, the detrimental effects of *P. gingivalis* LPS on foetal

development were not induced by activation of the inflammatory response. In fact, we showed in our ex vivo experiment that during normal pregnancy, cytokine production upon *P. gingivalis* LPS exposure is balanced towards a more anti-inflammatory ratio as compared with the non-pregnant situation. The exact mechanisms and implications of this pregnancy-related adaption of the inflammatory response to bacteria or LPS during pregnancy are intriguing and need further investigation.

At this moment there is no evidence to substantiate that periodontal treatment during pregnancy reduces the risk on pregnancy complications, except possibly for a subgroup of women with high risk of preterm birth. However, irrespective of an aetiological role of periodontitis in pregnancy complications, the state of pregnancy increases the susceptibility to gingival inflammation. Therefore, early detection and treatment of periodontal diseases prior to or during pregnancy remain unquestionably of benefit to the mother and should be performed at all time.

Nederlandse samenvatting

Samenvatting

In de inleiding *(hoofdstuk 1)* wordt kort de pathofysiologie van parodontitis, de mogelijke systemische consequenties van de parodontale infectie, alsook de vermeende rol van parodontitis in zwangerschapscomplicaties beschreven, met een extra focus op pre-eclampsie.

In 2003 is voor het eerst gesuggereerd dat parodontitis tijdens de zwangerschap mogelijk ook een risicofactor voor pre-eclampsie zou kunnen zijn. Pre-eclampsie is een ernstige, levensbedreigende aandoening die zich kan openbaren vanaf de 20ste week van de zwangerschap met hypertensie en proteinurie. Er is groeiend bewijs dat een chronische infectie (en de ontstekingsreactie hierop) een belangrijke etiologische factor is in de pathogenese van pre-eclampsie. Mogelijk draagt parodontitis, als een chronische infectie, bij aan de al toegenomen pro-inflammatoire staat van de normale zwangerschap, met pre-eclampsie als gevolg. Het is ook mogelijk dat paropathogene bacteriën of bacteriële producten vanuit de mondholte via de bloedbaan de placentaire en/of foetale weefsels bereiken en ter plaatse een ontstekingsproces induceren. Ten tijde van de aanvang van dit proefschrift was er weinig bewijs voor een relatie tussen parodontitis en pre-eclampsie. Het overkoepelende doel van dit proefschrift was dan ook om de aard van deze relatie te onderzoeken, in het bijzonder de epidemiologische associatie, het effect van parodontale therapie op het aantal pre-eclamptische zwangerschappen alsook pathologische mechanismen te onderzoeken die een relatie zouden kunnen verklaren.

De studie die wordt beschreven in *hoofdstuk 2* was gericht op het systematisch beoordelen van het beschikbare wetenschappelijke bewijs voor een verband tussen parodontitis en pre-eclampsie. Daartoe werd gezocht in de geautomatiseerde gegevensbestanden van MEDLINE via PubMed (1969-augustus 2010), EMBASE (1974-augustus 2010) en CINAHL (2003-augustus 2010). Relevante humane studies, gepubliceerd in het Engels, Duits, Frans, Spaans en Nederlands werden opgenomen. De methodologische kwaliteit van de primair geselecteerde studies werd door twee onderzoekers onafhankelijk van elkaar beoordeeld, met behulp van kwaliteitsbeoordelingsformulieren van de Nederlandse Cochrane Collaboration. Van de primair geselecteerde onderzoeken voldeden 12 observationele studies en drie gerandomiseerde, gecontroleerde studies (RCT's) aan de vooraf opgestelde inclusie- en exclusiecriteria. Acht van de observationele studies vonden een positieve associatie, terwijl vier studies geen associatie konden aantonen. De meest opvallende associatie werd gevonden tussen parodontitis en vroege pre-eclampsie (<34 weken zwangerschap). Het bleek echter moeilijk om de studies met elkaar te vergelijken, als gevolg van variaties in de gebruikte definities van parodontitis en pre-eclampsie, het tijdstip van parodontaal onderzoek en het ontbreken van voldoende controle voor confounders, zoals ethniciteit. Aangezien

studies die de parodontale status beoordeelden bij binnenkomst van de patiënt, d.w.z. voor pre-eclampsie was opgetreden, geen verband laten zien, is het onduidelijk of parodontitis een causale rol bij pre-eclampsie speelt. Geen van de geïncludeerde RCT's rapporteerde een reductie in pre-eclamptische zwangerschappen na parodontale therapie uitgevoerd rondom 20 weken zwangerschap. De meta-analyse toonde een aan parodontitis gerelateerd gecombineerd relatief risico op pre-eclampsie van 1.0 (95% CI 0.78-1.28). Er kan worden geconcludeerd dat parodontale therapie uitgevoerd rondom 20 weken zwangerschap geen positief effect heeft op zwangerschapsuitkomsten.

Gezien het feit dat in *hoofdstuk 2* de variatie in gebruikte definities voor parodontitis en pre-eclampsia, alsook ethniciteit mogelijk de uitkomsten hebben verstoord, is in de patiënt-controle studie zoals beschreven in *hoofdstuk 3*, de parodontale conditie en subgingivale plaque samenstelling van 17 Kaukasische vrouwen met een recent doorgemaakte pre-eclamptische zwangerschap vergeleken met de parodontale conditie van 35 Kaukasische vrouwen met een ongecompliceerd verlopen zwangerschap. Om consistentie zoveel mogelijk te garanderen zijn de parodontale definities gebruikt zoals eerder beschreven in de studie van Boggess et al (2003). Een volledig parodontaal onderzoek in de gehele mond werd uitgevoerd om de parodontale conditie vast te stellen. Subgingivale plaque monsters werden onderzocht op de aanwezigheid van zeven van de meest belangrijke paropathogenen. Ernstige parodontitis werd aangetroffen in 82% van de pre-eclamptische vrouwen en in 37% van de vrouwen uit de controlegroep. Na correctie voor leeftijd, BMI, roken en opleidingsniveau bleek ernstige parodontitis geassocieerd met pre-eclampsie (gecorrigeerde OR 7.9, 95% CI 1.9-32.8). De paropathogene bacterie *M. micros* (volgens recente taxonomie: *P. micra*) was verhoogd aanwezig in de onderzoeksgroep, terwijl *C. rectus* in hogere aantallen aanwezig was in de controlegroep. De resultaten laten zien dat de parodontale conditie van Kaukasische vrouwen met een recente geschiedenis van pre-eclampsie slechter is dan de parodontale conditie van vrouwen met een ongecompliceerde zwangerschap.

Om te onderzoeken of parodontitis een causale rol speelt in zwangerschaps-complications, is in *hoofdstuk 4* een experimentele dierstudie uitgevoerd, waarin is gekeken naar het effect van infusie van *P. gingivalis* LPS op zwangere ratten. In deze studie is onderzocht of de meest virulente component van *P. gingivalis*, het LPS, net zoals LPS van *E. coli*, zwangerschapscomplicaties, inclusief pre-eclamptische kenmerken, kon induceren in de rat. Voor dit doel zijn twee experimenten uitgevoerd. In experiment 1 werden zwangere ratten op dag 14 van de zwangerschap geïnfundeerd met oplopende doseringen van *P. gingivalis* LPS (0.0-50.0 µg/kg bw LPS; n=2/3 per dosering). De volgende parameters werden gedocumenteerd: maternale intra-arteriële bloeddruk, albumine-uitscheiding in de urine, placentair en foetaal gewicht en het aantal foetale

resorpties. In experiment 2 werden zwangere en niet-zwangere ratten geïnfundeerd met 10.0 µg/kg bw *P. gingivalis* LPS (de dosis die in experiment 1 de hoogste bloeddruk induceerde en het foetale gewicht reduceerde). Controle-ratten werden geïnfundeerd met fysiologisch zout (n=7/9 per groep). Naast de in experiment 1 onderzochte parameters, werden ook aantallen leukocyten in perifeer bloed en tekenen van ontsteking in de nier en placenta geëvalueerd. De resultaten van experiment 1 laten zien dat infusie met *P. gingivalis* LPS in zwangere ratten leidde tot verhoogde bloeddruk, een toename in het aantal foetale resorpties en een afname in placentair gewicht bij de meeste doseringen. Er was echter geen afname in foetaal gewicht, noch ontwikkelden de ratten albuminurie. Deze resultaten werden bevestigd in experiment 2 na infusie met 10.0 µg/kg bw *P. gingivalis LPS*. Echter, het aantal foetussen dat kleiner dan 2x de standaard deviatie (2 SD) van het normale gemiddelde was (4.75 ± 0.5 g; d.w.z. 3.75 gr), bleek significant hoger in de *P. gingivalis* LPS geïnfundeerde groep dan in de controle groep. Er werden geen tekenen van gegeneraliseerde ontsteking waargenomen en ook waren er geen indicaties van ontsteking in de nier en placenta. Gezien het feit dat er geen effect van *P. gingivalis* LPS op de bloeddruk bij niet-zwangere ratten was, is de toename in bloeddruk specifiek voor de zwangere ratten. Er kan worden geconcludeerd dat *P. gingivalis* LPS effect heeft op zwangerschap door het induceren van hypertensie en de placentaire en foetale groei negatief beïnvloedt alsook het aantal foetale resorpties doet toenemen. *P. gingivalis* LPS speelt daarom mogelijk een rol bij zwangerschapscomplicaties geïnduceerd door parodontitis. *P. gingivalis* LPS lijkt, in tegenstelling tot LPS van *E. coli*, geen rol te spelen in de pathogenese van pre-eclampsie, omdat er geen tekenen van albuminurie of ontsteking optraden.

In *hoofdstuk 5* is vervolgens gekeken of het verschil in effect van *P. gingivalis* LPS en *E. coli* LPS op de zwangerschap het gevolg zou kunnen zijn van verschillen in cytokineproductie na herkenning door leukocyten. Voor dit doel is bloed van 16 gezonde zwangere vrouwen en 15 gezonde niet-zwangere vrouwen gestimuleerd met bacteriën en LPS van *P. gingivalis* ATCC 33277 en *E. coli* in een ex-vivo laboratorium experiment. Na stimulatie is de cytokineproductie gemeten (TNF-α, IL-1β, IL-6, IL-12 and IL-10) met behulp van Luminex en is de TLR2/TLR4 expressie op monocyten vastgesteld met behulp van flow cytometrie. Tevens zijn pro-/anti-inflammatoire ratios (IL-12/IL-10, TNF-α/IL-10 and IL-6/IL-10) berekend. Stimulatie met *P. gingivalis* bacteriën/LPS induceerde, zowel in zwangere alsook in niet-zwangere vrouwen, lagere cytokineproductie en lagere pro-inflammatoire ratios dan *E. coli* bacteriën/LPS, met uitzondering van de IL-6/IL-10 ratio die was verhoogd na *P. gingivalis* bacteriën/LPS stimulatie. Er was tevens een effect van de zwangerschap op de cytokineproductie: in het bloed van zwangere vrouwen werd een lagere concentratie van IL-12 gemeten na stimulatie met *E. coli* bacteriën en een lagere IL-6 concentratie na stimulatie met *P.*

gingivalis bacteriën dan in het bloed van niet-zwangere vrouwen. Overeenkomstig was de productie van IL-12 en TNF-α in het bloed van zwangere vrouwen na stimulatie met LPS van *E. coli* en de productie van *IL-6* na stimulatie met *P. gingivalis* LPS lager dan in het bloed van niet-zwangere vrouwen. Het percentage TLR2 positieve monocyten was lager tijdens de zwangerschap dan in de niet-zwangere situatie. De lagere cytokine-productie en lagere pro-inflammatoire ratio na stimulatie met *P. gingivalis* vs. *E. coli* tijdens de zwangerschap verklaart mogelijk de verschillen in respons na bacteriële herkenning in de in-vivo situatie en suggereert dat tijdens de zwangerschap, vrouwen gevoeliger zijn voor de effecten van cytokines zoals IL-12, TNF-α en IL-6.

Meerdere studies hebben laten zien dat er variatie is in virulentie tussen verschillende *P. gingivalis* serotypen. Dit is mogelijk het gevolg van verschil in cytokineproductie na herkenning van de bacteriën of het LPS door ontstekingscellen van de gastheer. Om de virulentiefactoren van *P. gingivalis* verder te onderzoeken is in *hoofdstuk 6* een experiment uitgevoerd waarin is onderzocht in hoeverre cytokineproductie van leukocyten verschilt na stimulatie met bacteriën en geïsoleerd LPS van twee verschillende *P. gingivalis* serotypen. Voor dit doel werd de Mono Mac 6 (MM6) cellijn, alsook bloedmonsters van 15 gezonde vrouwen gestimuleerd met bacteriën en LPS van de ongekapselde *P. gingivalis* ATCC 33277 (K^-) en de gekapselde *P. gingivalis* W50 (K1). In experiment 1 werd de productie van TNF-α van de MM6 cellijn vastgesteld na stimulatie met bacteriën en LPS van beide *P. gingivalis* soorten met gebruikmaking van flow cytometrie. In experiment 2 werden de effecten van bacteriën of LPS van beide *P. gingivalis* serotypen op de productie van TNF-α, IL-1β, IL-6, IL-12 en IL-10 in bloed onderzocht met gebruikmaking van Luminex. Voor beide experimenten werden bacteriën en geïsoleerd LPS van *Escherichia coli* gebruikt als referentie. Beide *P. gingivalis* serotypen induceerden een lagere cytokineproductie dan *E. coli*, met uitzondering van IL-6 na stimulatie met *P. gingivalis* K1 bacteriën. Stimulatie met bacteriën van *P. gingivalis* K1 induceerde een hogere cytokineproductie dan stimulatie met bacteriën van *P. gingivalis* K^-. In tegenstelling, *P. gingivalis* K1 LPS induceerde een lagere cytokineproductie dan *P. gingivalis* K^- LPS. Blijkbaar zijn andere bacteriële producten dan het LPS verantwoordelijk voor de hogere cytokineproductie na stimulatie met *P. gingivalis* K1 in vergelijking tot *P. gingivalis* K^- bacteriën. De hogere cytokineproductie na stimulatie met bacteriën van *P. gingivalis* K1 draagt wellicht bij aan de virulentie-eigenschappen van dit serotype, omdat een toename in de productie van cytokines mogelijk leidt tot de recrutering van meer leukocyten naar het gebied van de infectie, waardoor het lokale ontstekingsproces wordt versterkt.

Tot slot worden in *hoofdstuk 7* de belangrijkste onderzoeksresultaten bediscussieerd en perspectieven voor toekomstig onderzoek gegeven. Uit de observationele studies,

inclusief de door ons uitgevoerde patiënt-controle studie, kan worden geconcludeerd dat er verband lijkt te bestaan tussen parodontitis en sommige vormen van pre-eclampsie. Deze associatie is het duidelijkst bij vroege-pre-eclampsie. Het is echter de vraag of parodontitis causaal betrokken is in de pathogenese van pre-eclampsie. Studies die een parodontale screening hebben uitgevoerd rond 20 weken van de zwangerschap (d.w.z. voordat de klinische symptomen van pre-eclampsie zich openbaarden) lieten geen verband zien tussen de beide aandoeningen. Ook parodontale behandeling, uitgevoerd rond 20 weken van de zwangerschap, leidde niet tot een reductie van het risico op pre-eclampsie. Het is dus onduidelijk of de parodontale infectie pre-eclampsie induceert of vice versa, of dat een tot nu toe onbekende, mogelijk genetische, factor ten grondslag ligt aan beide aandoeningen. Het kan echter niet worden uitgesloten dat parodontale behandeling rond 20 weken van de zwangerschap te laat is om translocatie van paropathogene bacteriën, zoals *P. gingivalis*, in de maternale circulatie en naar de foeto-placentaire weefsels te voorkomen.

De resultaten van het dierproefexperiment bevestigen eerdere bevindingen dat LPS van *P. gingivalis* een oorzakelijke bijdrage kan leveren aan het ontstaan van zwangerschapscomplicaties zoals een laag geboortegewicht en miskramen, in deze studie gerepresenteerd als foetale resorpties in de rat. Het LPS lijkt echter geen rol te spelen in de pathogenese van pre-eclampsie. Hoewel er een milde verhoging van de maternale bloeddruk werd gevonden, resulteerde infusie met LPS van *P. gingivalis* niet tot albuminurie of een gegeneraliseerde ontsteking. De foetale pathologie alsook de stijging in bloeddruk lijken van placentaire oorsprong zijn, omdat infusie met *P. gingivalis* LPS het placentaire gewicht negatief beïnvloedde. *P. gingivalis* LPS kan mogelijk de placentaire groei direct hebben beïnvloed door het induceren van apoptose van de trofoblast. Dit kan vervolgens hebben geleid tot een afgenomen toevoer van zuurstof en voeding naar de foetus, met als gevolg foetale groeivertraging. De milde stijging in bloeddruk kan ook een compensatiemechanisme van de moeder zijn geweest om de verstoorde zuurstof- en voedingstoevoer naar de foetus te herstellen. Het exacte mechanisme waarmee *P. gingivalis* LPS een stijging in maternale bloeddruk en placentaire en foetale groeivertraging heeft geïnduceerd blijft onopgehelderd in ons experiment en behoeft nader onderzoek.

De in-vitro en ex-vivo bloed experimenten laten zien dat zowel bacteriën als ook LPS van *P. gingivalis* een lagere pro-inflammatoire capaciteit hebben en een lagere cytokineproductie induceren dan *E. coli*. Dit verklaart mogelijk waarom in ons ratexperiment infusie met *P. gingivalis* LPS, in tegenstelling tot *E. coli* LPS, geen pre-eclampsie-achtige kenmerken induceerde. Het is opmerkelijk dat ondanks het feit dat zwangere vrouwen extreem gevoelig zijn voor endotoxine, de cytokineproductie in het bloed van zwangere vrouwen na de diverse stimulaties gelijk of lager was dan in het bloed van niet-zwangere vrouwen. Dit zou kunnen wijzen op een staat van endotoxine-

tolerantie van monocyten tijdens de zwangerschap en suggereert dat zwangere vrouwen gevoeliger zijn voor de effecten van cytokines. Vervolgonderzoek moet uitwijzen welke mechanismen verantwoordelijk zijn voor de lagere cytokineproductie na LPS stimulatie tijdens de zwangerschap.

Dit proefschrift toont aan dat er mogelijk een associatie is tussen parodontitis en sommige vormen van pre-eclampsie in observationele studies. Deze associatie was het meest duidelijk met vroege pre-eclampsie. Echter, parodontale therapie uitgevoerd tijdens de zwangerschap leidde niet tot een reductie in het aantal pre-eclamptische zwangerschappen. Ook induceerde *P. gingivalis* LPS in zwangere ratten geen pre-eclampsie-achtige verschijnselen of een gegeneraliseerde systemische ontstekingsreactie en werd er geen sterke pro-inflammatoire cytokineproductie opgewekt door *P. gingivalis* bacteriën of LPS in de in-vitro en ex-vivo experimenten. Het lijkt dan ook niet waarschijnlijk dat parodontitis een causale rol speelt in de pathogenese van pre-eclampsie.

De parodontale infectie speelt mogelijk een rol bij laag geboortegewicht en miskramen in de aanwezigheid van *P. gingivalis LPS*, zoals is aangetoond in het ratexperiment. Het is van belang om het exacte mechanisme te ontrafelen waarmee *P. gingivalis LPS* interfereert met de zwangerschap. Opmerkelijk is dat, in tegenstelling tot de algemene hypothese, de schadelijke effecten van *P. gingivalis LPS* op de foetale ontwikkeling niet werden veroorzaakt door een geactiveerde ontstekingsreactie. Ook ons ex-vivo experiment laat zien dat blootstelling aan *P. gingivalis LPS* tijdens de normale zwangerschap, in vergelijking tot de niet-zwangere situatie, een verschuiving in cytokineproductie teweeg brengt richting een meer anti-inflammatoire ratio. De exacte mechanismen en implicaties van deze aan de zwangerschap gerelateerde aanpassing van de ontstekingsreactie op bacteriën of LPS tijdens zwangerschap zijn intrigerend en dienen nader te worden onderzocht.

Op dit moment is er geen bewijs om te onderbouwen dat parodontale behandeling tijdens de zwangerschap het risico op zwangerschapscomplicaties reduceert, behalve mogelijk bij een subgroep van vrouwen met een hoog risico op vroeggeboorte. Echter, los van de mogelijkheid of parodontitis een etiologische rol speelt in zwangerschapscomplicaties, neemt tijdens de zwangerschap de gevoeligheid voor het ontwikkelen van parodontale ontstekingen toe. Om die reden blijft vroege detectie en behandeling van parodontale aandoeningen voorafgaand en tijdens de zwangerschap onbetwist van belang voor de moeder en dient te allen tijde te worden uitgevoerd.

Dankwoord

Dankwoord

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Curriculum vitae

Alina Kunnen was born on 17 March 1961 in Haarlem, the Netherlands. After finishing secondary school in 1979, she part-time studied graphic design and started a career in graphic art design in Amsterdam. In 1985 she worked in Pakistan (Karachi, Dar-Ul-Sukun) and emigrated to Spain (Tenerife, Canary Islands) in the same year. From 1987 to 1999 she was art director and owner of Alinea Typografie, a publicity agency in Amsterdam. After moving to the northern part of the Netherlands, she decided to make a career switch and started studying Oral Hygiene at the Hanze University of Applied Sciences/ Hanzehogeschool Groningen in 1999. She graduated in 2002 with the distinction cum laude. In 2002 she started a professional career as an instructor/lecturer at the Center for Dentistry and Oral Hygiene in Groningen and combined this with her private practice for Oral Hygiene in Joure, Friesland until 2007. She started her PhD research which led to the present thesis in 2005, and since then, she is combining research with being a lecturer in Periodontology and semester coordinator at the Center for Dentistry and Oral Hygiene, University of Groningen, University Medical Center Groningen, the Netherlands.

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