

Gingival changes during pregnancy: II. Influence of hormonal variations on the subgingival biofilm

Ana Carrillo-de-Albornoz,
Elena Figuero, David Herrera and
Antonio Bascones-Martínez

Department of Periodontology, School of
Dentistry, Complutense University of Madrid,
Spain

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Abstract

Aim: To determine whether the exacerbated gingival inflammation that develops in pregnant women is related to a change in the subgingival biofilm induced by the increase in hormone levels during pregnancy.

Material and Methods: This open cohort study included 48 pregnant and 28 non-pregnant women without periodontitis. Pregnant women were evaluated in the first, second and third trimester and at 3 months after delivery. Non-pregnant women were evaluated twice, with a 6-month interval, assessing microbiological, clinical and hormonal variables at each visit. Total anaerobic counts and frequency of detection and proportions were calculated. The Friedman test with the Bonferroni correction was used for intra-group comparisons and Mann–Whitney *U*-tests for inter-group assessment. Correlations were analysed by means of Spearman's rank correlation coefficient.

Results: Proportions of the subgingival periodontal pathogens did not differ throughout pregnancy, although significant differences were found for all the pathogens after delivery. *Porphyromonas gingivalis*-positive patients presented an increase in gingival inflammation ($p < 0.001$) that was not related to plaque. Correlations were found between maternal hormone levels and *P. gingivalis* and *Prevotella intermedia*.

Conclusion: Qualitative differences in periodontal pathogens were found from pregnancy to post-partum. Patients harbouring *P. gingivalis* presented and increased gingival inflammatory status.

Key words: *Aggregatibacter actinomycetemcomitans*; biofilm; microbiology; *Porphyromonas gingivalis*; pregnancy gingivitis; *Prevotella intermedia*

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Pregnancy gingivitis, defined as the gingival inflammation initiated by plaque

and exacerbated by endogenous sex steroid hormones (Mariotti 1999), is a common disease that affects 36–100% of pregnant women (Maier & Orban 1949, Loe & Silness 1963, Jensen et al. 1981). The influence of endogenous sex hormones on the periodontium is recognized in the currently accepted periodontal disease classification under the category of “dental plaque-induced gingival diseases modified by the endocrine system”, including gingivitis associated with puberty, menstruation and pregnancy (Armitage 1999).

The clinical features of pregnancy gingivitis do not differ from those of

common gingivitis, except for the tendency to develop severe signs of gingival inflammation without associated changes in plaque levels (Loe & Silness 1963, Silness & Loe 1964, Hugoson 1970, Arafat 1974, Chaikin 1977, O'Neil 1979a, Tilakaratne et al. 2000, Gürsoy et al. 2008). Most reports show an increase in the incidence of pregnancy gingivitis (Loe & Silness 1963, Silness & Loe 1964, Loe 1965, Cohen et al. 1969, 1971, Hugoson 1970, 1971, Arafat 1974, Samant et al. 1976, O'Neil 1979b, Zaki et al. 1984, Miyazaki et al. 1991, Muramatsu & Takaesu 1994, Machuca et al. 1999, Tilakaratne et al.

Conflict of interests and source of funding statement

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2000, Yalcin et al. 2002b, Gürsoy et al. 2008), although not all women present the same inflammatory pattern and cases can range from mild inflammation to severe hyperplasia, pain and profuse bleeding (Samant et al. 1976, Thomson & Pack 1982, Güncü et al. 2005). Although pregnancy gingivitis is not related to the level of plaque, it requires a minimum amount and does not develop in pregnant woman with excellent plaque control (Arafat 1974, Chaikin 1977). The most frequently affected areas appear to be the anterior sextants of the oral cavity, especially inter-proximal sites (Löe & Silness 1963, Raber-Durlacher et al. 1994). Pregnancy gingivitis is characteristically self-limiting and abates post-partum with the decline in hormone production (Hugoson 1971). A further characteristic of this entity is that it carries no risk of developing periodontitis, despite the inflammatory status developed (Cohen et al. 1969, Machuca et al. 1999, Tilakaratne et al. 2000, Lief et al. 2004).

Although exacerbated gingival inflammation in pregnant women is clinically and histologically well documented, its aetiology has not yet been clearly established and it is not known why only some pregnant women develop frank signs of gingival inflammation (Amar & Chung 1994, Mariotti 1994, Laine 2002, Mascarenhas et al. 2003, Mealey & Moritz 2003). Four potential mechanisms have been proposed, including an increase in vascular permeability (Lindhe et al. 1967, Lindhe & Branemark 1967a, b, 1968a, b, Hoffman et al. 1981); a change to a more susceptible gingival phenotype (Dyer et al. 1980, Mariotti 2005); immune system depression (O'Neil 1979b, Lopatin et al. 1980, Miyagi et al. 1993, Raber-Durlacher et al. 1993, Lapp et al. 1995, 2003); and changes in the sub- or the supragingival biofilm (Kornman & Loesche 1980, Jensen et al. 1981, Raber-Durlacher et al. 1994).

One of the most solidly based hypotheses is a possible change in the subgingival biofilm. However, limited data exist on the composition of the subgingival microbiota during pregnancy. Direct and indirect aetiopathogenic pathways have been proposed. According to the former, the increase in hormone levels would promote the overgrowth of specific pathogenic bacteria that are responsible for the increased gingival inflammation. Thus, Kornman & Loesche (1982) demonstrated that *Prevotella intermedia* and *Porphyromonas gingivalis* (to a lesser extent) can replace progesterone or oes-

tradiol with vitamin K, an essential growth factor for this bacteria (Gibbons & MacDonald 1960). In the indirect pathway, the greater exposure to sex steroid hormones would transform the gingiva into a more susceptible environment due to greater gingival probing depths (Miyazaki et al. 1991), a higher gingival crevicular flow rate (Lindhe & Branemark 1968a, b), lower keratinization of the marginal gingival epithelium and reduced immunoresponsiveness, which would together favour the entry of more pathogenic bacteria into the subgingival biofilm. Accordingly, the presence of the pathogen would be the consequence and not the cause of the condition.

Various cross-sectional and longitudinal studies have evaluated the effects of sex steroid hormones on the subgingival biofilm, but the results have been inconclusive and this issue remains controversial. Several authors have proposed the increase in *P. intermedia* during pregnancy as an aetiological factor in pregnancy gingivitis. Kornman & Loesche (1980) were the first to describe an increase in *P. intermedia* during the second trimester concomitant with an increase in gingival inflammation. A cross-sectional study by Jensen et al. (1981) found a 55-fold higher level of *P. intermedia* in pregnant versus non-pregnant women. An association between higher *P. intermedia* levels and increased gingival inflammation was also reported using an experimental gingivitis model in pregnant patients, whereas the elevated inflammation status observed during pregnancy was not observed post-partum using the same model (Raber-Durlacher et al. 1994). However, other authors found no microbiological differences between pregnant and non-pregnant women (Jonsson et al. 1988, Gürsoy et al. 2009).

Controversy also surrounds the gingivitis related to hormonal alterations associated with puberty, contraceptive intake or the menstrual cycle. Qualitative changes in the biofilm, especially the overgrowth of *P. intermedia* (Delaney et al. 1986, van Oosten et al. 1988, Mombelli et al. 1990, Nakagawa et al. 1994), have also been implicated in the increased severity of gingivitis in these situations. Other microbiological findings, such as the increase in *Capnocytophaga* sp. before the increase in *P. intermedia*, have implicated this bacterial species in the onset of puberty gingivitis (Mombelli et al. 1990). However, some authors found no relevant

changes in the biofilm (Yanover & Ellen 1986, Gusberti et al. 1990).

After clinical, hormonal and immunological data were analysed in the first part of the present longitudinal study (Part I, Figuero et al. 2009), the objective of this second part (Part II) was to test the hypothesis that pregnancy induces changes in the subgingival biofilm that are responsible for the exacerbated gingival inflammation developed in pregnant women. The specific study objectives were (1) to longitudinally and prospectively evaluate any change in microbiological status over the three trimesters of pregnancy; (2) to assess whether the presence of periodontal pathogens is associated with an increase in gingival inflammation; and (3) to correlate microbiological patterns with clinical and hormonal status. In a secondary analysis, the microbiological results were compared with findings at the post-partum visit and with a group of non-pregnant women.

Material and Methods

Experimental design

This was an open cohort prospective study with a parallel design and a 9-month follow-up. All participants signed their informed consent to participation in the study, which was approved by the Research and Ethics Committee of San Carlos University Hospital (Madrid).

Population screening

Based on information from previous reports on pregnancy gingivitis (Cohen et al. 1969, Tilakaratne et al. 2000), a difference of 0.15 in the gingival index (GI) (Löe & Silness 1963) was considered as a reference. Power calculation was performed with the Sample Power 2.0 software pack. This analysis indicated that with 45 pregnant subjects, the study would have 80% power to detect a 0.15 difference in the GI during pregnancy, with α set at 0.05.

Therefore, taking into account potential drops-out, sample size was established at 60 pregnant women. Potential subjects were consecutively recruited from the Obstetrics Department of José Marvá Hospital (pregnant group) and from the Reception Department of the School of Dentistry of Madrid Complutense University (non-pregnant group). All subjects underwent a periodontal examination, including full-

mouth evaluation of probing pocket depth, clinical attachment level and bleeding on probing (six sites per tooth). After the periodontal diagnosis was established, all subjects who fulfilled the inclusion/exclusion criteria were invited to participate in the study until the desired sample sizes were reached.

Inclusion/exclusion criteria

Inclusion criteria were (i) age 20–35 years and (ii) presence of ≥ 20 natural teeth in mouth excluding third molars. Exclusion criteria were (i) diagnosis of chronic/aggressive periodontitis (Armitage 1999); (ii) presence of acute dental or periodontal disease; (iii) smoking habit; (iv) presence of systemic disease and/or medication affecting the periodontium; and (v) receipt of systemic antibiotic treatment or dental prophylaxis in the previous 6 months.

In addition to the above inclusion/exclusion criteria, pregnant women were included if they were in the 12th to 14th week of pregnancy in the first visit and non-pregnant women were excluded if they were taking contraceptive drugs or if they were pregnant or planning to be so.

Study design

Data were gathered on the pregnant women at four visits: at the end of the first trimester (12–14 weeks of pregnancy), second trimester (23–25 weeks of pregnancy) and third trimester (33–36 weeks of pregnancy) and at 3 months post-delivery. Data on non-pregnant women were collected at two visits 6 months apart, matching the first and the third visits of the pregnant group. The hormone status of the menstrual cycle was controlled by scheduling visits for the non-pregnant women during the luteal phase (days 17–21) of the cycle (Zaki et al. 1984).

Primary outcome (microbiological) variables and secondary outcome (clinical and hormone) variables were evaluated at each visit (Fig. 1), collecting (in this order): saliva sample for hormone analysis, plaque index (PII), microbiological sample and, finally, GI. After all evaluations and sampling, patients received oral hygiene instructions, a toothbrush (Vitis access[®], Dentaïd, Barcelona, Spain) and a dentifrice (Colgate Total[®], Palmolive[®], Piscataway, NJ, USA). At the end of the study, all subjects received a dental prophylaxis.

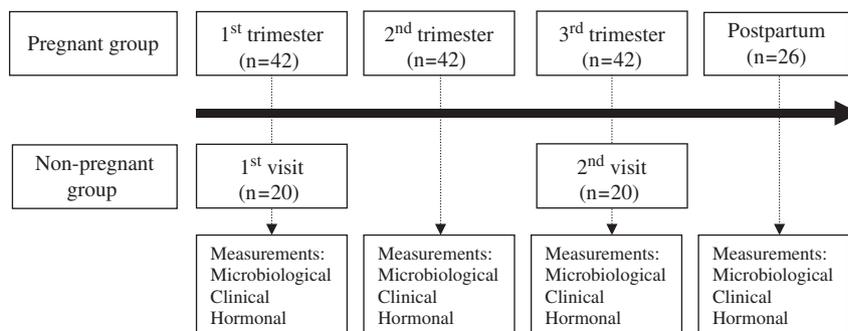


Fig. 1. Research outline. Pregnant patients made four visits and the non-pregnant group made two visits, corresponding in time to the first and the third trimesters of pregnancy. Microbiological, clinical and hormonal data were recorded at all visits.

Microbiological analysis

Bacterial sampling

A pooled subgingival sample was obtained from four sampled sites. At each visit, the sample was obtained from the four inter-proximal sites showing the most marked inflammation per quadrant, excluding those sites in which a periopaper strip (Harco, Irvine, CA, USA) was inserted for immunological analysis (described in Part I of the study, Figuero et al. 2009). After careful removal of supragingival plaque deposits, sampling sites were isolated with cotton rolls and gentle air drying, and two sterile #30 paper points (Zipperer[®], United Dental MFRS Inc., West Palm Beach, FL, USA) were then consecutively inserted into the depth of the sulcus and left in place for 10 s. All paper points were transferred to the same vial containing 1.5 ml of reduced transport fluid (RTF) (Syed & Loesche 1972) and maintained at 4°C until their inoculation within 24 h. All microbiological samples were taken by the same examiner (A. C.).

Culture procedures

Cultures were performed at the Laboratory of Oral Microbiology, School of Dentistry, Madrid Complutense University (Spain). Samples were vortexed for 30 s and 10-fold serially diluted in RTF, and 0.1 ml of each dilution was then plated on non-selective 5% horse blood agar plates (Oxoid no. 2, Oxoid Ltd., Basingstoke, UK) supplemented with haemin (5 mg/l) and menadione (1 mg/l) for determination of total anaerobic bacterial counts and specific periodontal pathogens (*P. intermedia/Prevotella nigrescens*, *P. gingivalis*, *Tannerella forsythia*, *Fusobacterium nucleatum*, *Parvi-*

monas micra, *Eikenella corrodens*, *Campylobacter rectus* and *Capnocytopaga* sp.). Total anaerobic counts were performed after 7–14 days of incubation by means of a stereo microscope (SDZ-PL, Kyowa Optical Co. Ltd., Hashimoto, Japan). Blood agar plates were incubated at 37°C in 80% N₂, 10% CO₂ and 10% H₂. The presence and numbers of the putative periodontal pathogens were recorded, confirming their identification by means of Gram staining and cell morphology, aero tolerance, production of catalase and other biochemical reactions (Rapid ID 32A, BioMerieux SA, Le-Balme-les-Grottes, France).

Samples were also plated on Dentaïd-1 medium (Alsina et al. 2001) for isolation and counting of *Aggregatibacter actinomycetemcomitans*. Dentaïd-1 plates were incubated in air with 5% CO₂ at 37°C for 2 days. Total *A. actinomycetemcomitans* counts were obtained, identifying the bacteria from their characteristic colony morphology (star-like inner structure), a positive catalase reaction with 3% hydrogen peroxide and a set of specific enzymes (APIZYM, BioMerieux SA).

Clinical examination

Full-mouth supragingival PII and GI were recorded at four sites per tooth (mesial, distal, buccal and lingual) with a CPC-12 periodontal probe (Hufriedy[®], Leimen, Germany) according to Silness & Loe (1964) and Loe & Silness (1963), respectively. All clinical parameters were recorded by the same examiner (E. F.).

Hormone-level measurement in saliva (progesterone and estradiol)

Unstimulated saliva was collected in a sterile glass tube for 2 min. (Tallon et al.

1984, Gann et al. 2001) after patients had rinsed their mouths with water and rested for 5 min. Samples were frozen and stored at -20°C until further analysis (Tallon et al. 1984, Zaki et al. 1984, Morishita et al. 1988, Meulenberg & Hofman 1989, Gann et al. 2001). Progesterone and oestradiol levels were determined by means of a competitive immunoenzymatic colorimetric method (DIA.METRA SRL, Foligno[®], Paciana-Perugia, Italy).

The material and methods used in this study were partially described in Part I (Figuro et al. 2009).

Data management and statistical analysis

Counts of the culture-identified pathogens were performed by direct counting of the selected colonies with a stereo microscope (SDZ-PL, Kyowa Optical Co. Ltd.) on suitable plates (with 30–300 colonies) and estimated in relation to the original sample. The total anaerobic counts were also calculated on blood agar plates. Results were expressed in colony-forming units per millilitre (CFU/ml). After identification, proportions of the different pathogens were calculated and expressed as a percentage of the total CFU.

A subject-level analysis was performed for each study parameter. As goodness of fit was not assumed due to the skewed distribution of the data, non-parametric tests were used for the microbiological analysis.

Intra-group differences in the counts and proportions of the periodontal pathogens over time were tested by means of non-parametric analysis of variance (ANOVA) Friedman's test. Post hoc comparisons were performed using Bonferroni's method.

The frequency of pathogen detection was obtained by transforming the results into categorical data and using the McNemar test to evaluate intra-group differences and the χ^2 -test to compare inter-group differences. The correlation between pairs of variables was evaluated using the non-parametric Spearman rank-correlation coefficient (correlation of microbiological with hormonal and clinical data).

Differences in the GI during pregnancy depending on the presence (positive patients)/absence (negative patients) of the different pathogens were evaluated using the Mann-Whitney *U*-test. Differences in the PII were also included in the subgroup analysis to control the effect of plaque on the GI.

To determine differences between pregnant and non-pregnant women, the Mann-Whitney *U*-test was used.

Statistical significance was established at the 95% confidence level. SPSS for Windows (SPSS Inc. version 16.0) was used for the data analyses.

Results

Study population

Sixty pregnant women were invited to participate in the study. Out of this sample, 48 agreed to participate and 42 complied with the three visits during the whole pregnancy. The reasons for the loss of patients to the study were miscarriage (three), pre-term birth (one), isolation for suspicion of tuberculosis (one) and personal reasons/no time available (one); their data were excluded from the analyses. The fourth post-partum visit was complied by 26 women, and the remaining 16 were lost for personal reasons (no time, loss of interest). No differences were found in any of the microbiological variables evaluated between the women who completed the follow-up and the 16 who withdrew after the delivery (non-parametric ANOVA Friedman's test; data not shown).

Thirty non-pregnant women were invited to participate in the study, and 28 of these were enrolled after voluntarily agreeing to participate. After 6 months of follow-up, eight patients were lost (four started taking oral contraceptives and four refused to continue because of no interest/no time), and their data were excluded from the analysis. No differences were found in the

microbiological results between the non-pregnant women who completed the follow-up and the eight who withdrew the second visit (non-parametric ANOVA Friedman's test; data not shown).

Microbiological results during pregnancy and post-partum

Total bacterial counts

No significant changes were observed in the total bacterial counts in the pregnant group during pregnancy or after delivery (Table 1).

Frequency of detection of pathogens

Table 2 shows the prevalence of the studied periodontal pathogens in the subgingival plaque samples from the pregnant women followed up throughout their pregnancy. The most frequently detected pathogen during pregnancy was *F. nucleatum* (range 88.10–97.62%), followed in descending order by *P. intermedia* (47.62–66.67%), *P. micra* (42.86–50.00%), *P. gingivalis* (35.71–40.48%), *C. rectus* (11.90–14.29%), *A. actinomycetemcomitans* (9.52–21.43%) and *T. forsythia* (2.38–9.52%).

The frequency of pathogen detection was relatively constant throughout the pregnancy and tended to decrease after delivery, when there was a significant reduction in *A. actinomycetemcomitans* ($p = 0.039$).

Proportions (%) of bacterial species

Table 3 summarizes the relative proportions of bacterial species isolated

Table 1. Total bacterial counts expressed in total colony-forming units (CFU) in pregnant and non-pregnant women

	Pregnant group			
	first trimester (<i>n</i> = 42)	second trimester (<i>n</i> = 42)	third trimester (<i>n</i> = 42)	post-partum (<i>n</i> = 26)
Mean counts	5.32×10^5	5.28×10^5	5.03×10^5	5.02×10^5
Standard deviation	8.07×10^5	8.30×10^5	8.32×10^5	9.50×10^5
	Non-pregnant group			
	baseline (<i>n</i> = 20)	6 months (<i>n</i> = 20)		
Mean counts	4.96×10^5	4.63×10^5		
Standard deviation	8.45×10^5	8.69×10^5		

Intra-group comparison (Friedman's test with Bonferroni's corrections).

Table 2. Frequency of detection of periodontal pathogens (percentage/absolute number related to group)

	Pregnant group			
	first trimester (n = 42)	second trimester (n = 42)	third trimester (n = 42)	post-partum (n = 26)
<i>Aggregatibacter actinomycetemcomitans</i>	14.29% (6)	9.52% (4)	21.43% (9)	7.69% (2)*
<i>Porphyromonas gingivalis</i>	35.71% (15)	40.48% (17)	38.10% (16)	26.92% (7)
<i>Prevotella intermedia</i>	61.90% (26)	66.67% (28)	47.62% (20)	57.69% (15)
<i>Tannerella forsythia</i>	9.52% (4)	2.38% (1)	7.14% (3)	7.69% (2)
<i>Parvimonas micra</i>	50.00% (21)	42.86% (18)	47.62% (20)	38.46% (10)
<i>Campylobacter rectus</i>	11.90% (5)	14.29% (6)	14.29% (6)	7.69% (2)
<i>Fusobacterium nucleatum</i>	97.62% (41)	92.86% (39)	88.10% (37)	80.77% (21)

	Non-pregnant group	
	baseline (n = 20)	6 months (n = 20)
<i>Aggregatibacter actinomycetemcomitans</i>	0.00% (0)	0.00% (0)
<i>Porphyromonas gingivalis</i>	10.00% (2)	5.00% (1)
<i>Prevotella intermedia</i>	30.00% (6)*	40.00% (8)
<i>Tannerella forsythia</i>	5.00% (1)	0.00% (0)
<i>Parvimonas micra</i>	30.00% (6)	30.00% (6)
<i>Campylobacter rectus</i>	10.00% (2)	20.00% (4)
<i>Fusobacterium nucleatum</i>	95.00% (19)	85.00% (17)

* $p < 0.05$ in intra-group comparisons.

Table 3. Longitudinal evaluation of the periodontal pathogens during pregnancy and at 3 months post-partum (intra-group comparison)

Bacterial species	Relative proportions (%) of pathogens during pregnancy (SD)			
	first trimester (n = 42)	second trimester (n = 42)	third trimester (n = 42)	post-partum (n = 26)
<i>Aggregatibacter actinomycetemcomitans</i>	0.30 (1.47)	1.02 (6.10)	1.22 (4.26)	0.08 (0.41)**
<i>Porphyromonas gingivalis</i>	5.69 (12.17)	4.55 (8.76)	8.12 (17.19)	2.74 (8.13)**
<i>Prevotella intermedia</i>	2.37 (5.13)	3.13 (8.80)	2.55 (9.91)	3.83 (8.64)*
<i>Tannerella forsythia</i>	0.46 (2.16)	0.01 (0.04)	0.41 (1.66)	0.03 (0.14)*
<i>Parvimonas micra</i>	2.66 (7.47)	3.27 (7.15)	2.06 (4.82)	1.95 (3.71)**
<i>Campylobacter rectus</i>	0.07 (0.29)	0.15 (0.65)	0.13 (0.62)	0.26 (1.12)**
<i>Fusobacterium nucleatum</i>	5.38 (7.52)	5.59 (5.24)	5.38 (7.09)	4.01 (4.04)**

Friedman's test with Bonferroni's corrections.

* $p < 0.05$; ** $p < 0.01$.

throughout pregnancy and post-partum. The studied pathogens exhibited a common pattern during pregnancy characterized by a progressive increase during the pregnancy, with a peak at either the second or the third trimester, depending on the pathogen. The non-parametric ANOVA Friedman's test failed to achieve statistically significant differences during pregnancy for any of the pathogens evaluated. However, changes from the third trimester to post-partum were significant for all the pathogens. *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *P. micra* and *F. nucleatum* demonstrated an abrupt clear decrease after delivery, whereas *P. intermedia* and *C. rectus* significantly increased

post-partum in comparison with the third trimester.

Proportions in positive samples of different bacterial species

The frequency of detection varied among patients for the different pathogens. In order to evaluate the microbiological pattern in positive samples, "positive" and "negative" patient subgroups were formed according to the presence/absence of the pathogen in the second trimester (Herrera et al. 2008) (Table 4).

The proportion of *A. actinomycetemcomitans* in patients harbouring this pathogen was 2.24% in the first trime-

ster, reached a peak in the second trimester (10.80%) and decreased to 5.69% in the third term ($p = 0.01$). Following the same pattern, the proportion of *P. micra* increased from 4.88% to 6.18% and 4.55% in the first, second and the third trimester, respectively. Changes in *P. micra* proportions were significant at the third trimester visit ($p < 0.05$).

No significant changes were observed in any of the remaining periodontal pathogens studied. The proportion of *P. gingivalis*-positive patients was 14.94% at the first trimester, slightly decreased to 11.25% during the second trimester and reached a peak of 20.07% at the third term ($p = 0.052$).

Table 4. Proportion in positive samples of periodontal pathogens during pregnancy

	Mean percentage (SD) of flora in positive patients			Friedman test <i>p</i> value	<i>p</i> -value (Bonferroni's correction)	
	first trimester	second trimester	third trimester		first versus second trimester	second versus third trimester
<i>Aggregatibacter actinomycetemcomitans</i>	2.24 (3.54)	10.80 (19.18)	5.69 (8.03)**	< 0.01	NS	0.01
<i>Porphyromonas gingivalis</i>	14.94 (16.05)	11.25 (10.80)	20.07 (22.43)	NS	NS	NS
<i>Prevotella intermedia</i>	3.99 (6.18)	5.47 (10.98)	5.43 (14.01)	NS	NS	NS
<i>Tannerella forsythia</i>	4.84 (5.96)	0.26 (0.00)	5.77 (3.17)	NS	NS	NS
<i>Parvimonas micra</i>	4.88 (9.81)	6.18 (9.30)	4.55 (6.32)*	<0.05	NS	<0.05
<i>Campylobacter rectus</i>	0.65 (0.66)	1.29 (1.58)	1.04 (1.51)	NS	NS	NS
<i>Fusobacterium nucleatum</i>	5.63 (7.56)	6.36 (5.13)	6.28 (7.28)	NS	NS	NS

Intra-group comparison (Friedman's test with Bonferroni's corrections).

p*<0.05; *p*<0.01.

Gingival inflammation in patients harbouring periodontal pathogens

A comparison between positive and negative patients for the different pathogens was made in order to detect differences in their GI. Changes in the PII were also evaluated to control the effect of plaque over the GI.

The bacterial species whose presence was associated with an increase in the GI were *P. gingivalis* and *P. intermedia*. Subjects who were positive for *P. gingivalis* had higher levels of gingival inflammation versus *P. gingivalis*-negative patients, regardless of their PII, in all trimesters of pregnancy. This increase was significant in the second (*p*<0.001) and the third trimesters (*p*=0.009), and no differences were found after delivery. The GI was 1.34 [standard deviation (SD) 0.34] in positive patients versus 0.98 (SD 0.45) in negative patients in the second trimester, and 1.35 (0.34) in positive patients versus 0.98 (0.46) in negative patients in the third trimester (Table 5).

Both the GI and the PII were higher in pregnant women harbouring *P. intermedia* than in those with negative culture for this pathogen (Table 6), with a significant difference in the second trimester (*p*=0.026). This tendency was also observed for *A. actinomycetemcomitans*-positive patients in the first trimester, although the difference in GI did not reach significance (*p*=0.053).

Correlations between clinical, hormonal and microbiological outcomes

The increase in salivary progesterone concentrations from the first to the second trimester was positively correlated with *P. gingivalis* (*r*=0.357, *p*=0.016). A

Table 5. Levels of plaque index (PII) and gingival index (GI) according to the presence [positive patients (+)]/absence [negative patients (-)] of *Porphyromonas gingivalis* during pregnancy (intra-group comparison)

	Gingival inflammation in patients harbouring <i>P. gingivalis</i> – mean (SD)			
	first trimester	second trimester	third trimester	post-partum
PII				
<i>P. gingivalis</i> +	0.78 (0.34)	0.74 (0.27)	0.75 (0.25)	0.79 (0.24)
<i>P. gingivalis</i> –	0.67 (0.38)	0.60 (0.36)	0.61 (0.32)	0.69 (0.30)
GI				
<i>P. gingivalis</i> +	1.10 (0.41)	1.34 (0.34)**	1.35 (0.34)**	0.90 (0.29)
<i>P. gingivalis</i> –	0.94 (0.44)	0.98 (0.45)	0.98 (0.46)	0.99 (0.40)

***p*<0.01.

Table 6. Levels of plaque index (PII) and gingival index (GI) according to the presence [positive patients (+)]/absence [negative patients (-)] of *Prevotella intermedia* throughout pregnancy (intra-group comparison)

	Gingival inflammation in patients harbouring <i>P. intermedia</i> – mean (SD)			
	first trimester	second trimester	third trimester	post-partum
PII				
<i>P. intermedia</i> +	0.76 (0.41)	0.77 (0.34)*	0.72 (0.33)	0.76 (0.30)
<i>P. intermedia</i> –	0.67 (0.38)	0.47 (0.21)	0.62 (0.26)	0.66 (0.26)
GI				
<i>P. intermedia</i> +	1.05 (0.44)	1.26 (0.42)*	1.26 (0.47)	0.91 (0.44)
<i>P. intermedia</i> –	0.92 (0.42)	0.94 (0.42)	1.01 (0.40)	1.04 (0.27)

**p*<0.05.

positive correlation was also found at this stage between GI exacerbation and increase in *P. intermedia* (*r*=0.345, *p*=0.020). From the second to the third trimester of pregnancy, oestradiol concentration was correlated with *P. gingivalis* (*r*=0.324, *p*=0.041).

Comparison between pregnant and non-pregnant women

The non-pregnant group showed no significant differences in microbiological

outcomes between the two visits (separated by a 6-month interval).

Total bacterial counts

The total bacterial counts tended to be higher in the pregnant versus non-pregnant women. The difference was only significant at the first-trimester visit, and no differences were found between the third-trimester visit of the pregnant women and the 6-month follow-up visit of the non-pregnant women (Table 1).

Frequency of detection of bacterial species

Table 2 describes the prevalence of the periodontal pathogens in subgingival plaque samples from the pregnant and non-pregnant groups, again comparing first-trimester and third-trimester results for the pregnant women with baseline and 6-month follow-up results for the non-pregnant women. In the first-trimester/baseline visit comparison, only the frequency of *P. intermedia* detection was significantly higher in the pregnant group (61.90%) than in the non-pregnant group (47.62%). In the third-trimester/6-month visit comparison, *A. actinomycetemcomitans* and *P. gingivalis* were more frequently present, with *A. actinomycetemcomitans* detected in 21.43% of the pregnant women but none of the non-pregnant group and *P. gingivalis* detected in 38.10% of the pregnant women and 5% of the non-pregnant women.

Proportions (%) of bacterial species

Qualitative differences were detected in the subgingival bacterial composition between the pregnant and the non-pregnant women, with higher proportions of periodontal pathogens in the pregnant versus non-pregnant women. Proportions of *P. intermedia* were significantly higher at the first-trimester visit in the pregnant group than at the first visit in the non-pregnant group. Proportions of *A. actinomycetemcomitans* and *P. gingivalis* were significantly higher at the third-trimester visit in the pregnant group than at the 6-month visit in the non-pregnant group.

Comparison of post-partum with non-pregnant women

At 3 months after delivery, the pregnant women did not significantly differ from the non-pregnant group in any of the pathogens evaluated.

Discussion

This open cohort longitudinal study was designed to assess whether pregnancy induces a change in the subgingival biofilm that could be responsible for the exacerbated gingival inflammation observed in pregnant women. In the present study, no changes were found in the subgingival microbiological profile throughout pregnancy, although significant differences were found for all

the periodontal pathogens after delivery. This implies a qualitative modification of the subgingival biofilm from pregnant to post-partum status. A worsening of the clinical parameters was also associated with the presence of *P. gingivalis* and *P. intermedia*, which were positively correlated with the salivary hormone levels.

To evaluate the exposure of gingival tissues to pregnancy changes, a cohort study was undertaken as it offers a better control of the confounding factors within observational studies (Stroup et al. 2000). The study population consisted of a group of pregnant women with no presence or history of periodontitis that was compared, in a secondary analysis, with a control group of non-pregnant women. To evaluate the subgingival microbiological pattern during pregnancy, the pregnant women were followed longitudinally throughout the three trimesters of pregnancy and were re-evaluated at 3 months after delivery, following the practice of previous studies that addressed the effect of pregnancy on the periodontium (Cohen et al. 1969, Cohen et al. 1971, Chaikin 1977, Tilakaratne et al. 2000, Yalcin et al. 2002, Gürsoy et al. 2008).

Culture was the method used to determine periodontal pathogens because of its accuracy and ability to simultaneously detect and quantify multiple bacterial species and reveal unexpected bacteria (Sanz et al. 2004). The limitations of culture include the higher detection threshold versus PCR and the difficulty of growing some periodontal pathogens, such as *T. forsythia* (Lau et al. 2004). Samples were taken at whole-mouth sites with the greatest inflammation, carefully evaluating anterior localizations, where pregnancy gingivitis typically develops (Løe & Silness 1963, Raber-Durlacher et al. 1993, 1994).

Within the limitations of the study, it is important to highlight the elevated dropout rate (especially after delivery), although the sample size was powerful enough to detect significant differences for all the pathogens in the comparison of the third trimester with the post-partum visit. Another remarkable weakness of the study was the lack of homogeneity between the pregnant and the non-pregnant groups in demographic characteristics and initial clinical status, which hampers the comparison between groups. The educational level of pregnant women in our study was lower than the non-pregnant group. As described previously,

the oral health status in pregnant women is related to the level of education, professional status and previous dental attendance (Machuca et al. 1999, Yalcin et al. 2002, Taani et al. 2003, Sarlati et al. 2004). To what extent these differences could have influenced the microbiological results in the comparison between pregnant and not pregnant patients has not been reported earlier.

Total bacterial counts

We first ruled out the possibility that an increase in the subgingival total bacterial count during pregnancy might be responsible for the clinical changes observed. No significant differences were observed in the total bacterial counts among the different trimesters of pregnancy, implying that no quantitative microbiological changes were responsible for the exacerbation in the gingival inflammation, which is in agreement with previous reports (Kornman & Loesche 1980, Jonsson et al. 1988, Yokoyama et al. 2008, Adriaens et al. 2009).

Frequency of detection of pathogens

Patients who harboured pathogens from the beginning of pregnancy maintained levels above the culture detection threshold until delivery, after which the frequency of detection decreased. The prevalence of *A. actinomycetemcomitans* during pregnancy ranged from 16.4% to 20.5%, higher than the 6.3% detected by culture in a sample of patients with gingivitis in Spain (Lau et al. 2004). Comparisons cannot be made with previous culture findings in pregnant women without periodontitis, because this pathogen was not included in the subgingival microbiological analyses (Kornman & Loesche 1980, Jensen et al. 1981, Jonsson et al. 1988, Muramatsu & Takaesu 1994, Raber-Durlacher et al. 1994). With checkerboard DNA–DNA hybridization technology, 25% of the tested sites were positive for *A. actinomycetemcomitans* (Adriaens et al. 2009). *A. actinomycetemcomitans* was not detected in our non-pregnant control group, taking into account that PII and GI were already low in this group at baseline.

P. gingivalis was detected in 40% of the pregnant women, similar to culture findings in the Spanish population with gingivitis (Lau et al. 2004), and higher than the 20% obtained by the Swiss group with checkerboard DNA–DNA hybridization (Adriaens et al. 2009).

Proportions of pathogens

Minor differences were observed in the composition of the subgingival microbiota during pregnancy, although significant differences emerged at the post-partum visit, where a generalized reduction of the pathogenic bacterial load was established. In this sense, the microbiological results corroborate our clinical findings (Part I, Figuero et al. 2009). A more pathogenic subgingival flora was associated with an increased gingival inflammation status during pregnancy. After delivery, the microflora underwent a qualitative change, and the frequency of detection, counts and percentages of pathogens declined sharply, as described previously (Kornman & Loesche 1982, Raber-Durlacher et al. 1994). This was clinically associated with a decrease of the GI despite the concomitant increase of the PII observed. Changes in the post-partum microflora have also been reported by means of checkerboard DNA–DNA hybridization for eight of 37 species analysed (*Eubacterium saburreum*, *F. nucleatum naviforme*, *F. nucleatum polymorphum*, *Leptotrichia buccalis*, *P. micra*, *Selenomonas noxia*, *Staphylococcus aureus* and *Streptococcus mutans*) (Adriaens et al. 2009).

Within the generalized reductions in periodontal pathogens, species such *P. intermedia* and *P. micra* suffered an overgrowth after delivery. Gürsoy et al. (2009) corroborate our results after reporting a significant increase of *P. intermedia sensu lato* 4–6 weeks after delivery. Muramatsu & Takaesu (1994) also observed an increase of *P. intermedia* proportions 1 month after delivery. Other recent studies have reported higher bacterial levels of *Actinomyces neuii*, *Bifidobacterium bifidum*, *Corynebacterium pseudogenitalis*, *Porphyromonas endodontalis*, *Porphyromonas bivia* and *Pseudomonas aeruginosa* in parous women with gingivitis, suggesting that gingivitis is common for a considerable time after delivery (Persson et al. 2008, 2009).

To avoid underestimation of the true impact of the pathogens in positive patients, especially in those with a lower frequency of detection, proportions in positive samples were analysed (Herrera et al. 2008). All the pathogens showed the highest proportions at the second term and slightly decreased at the third term, except for *P. gingivalis* and *T. forsythia*. This pattern, significant for *A. actinomycetemcomitans* and *P. micra*, is in agreement with previous

reports (Kornman & Loesche 1980, Adriaens et al. 2009, Gürsoy et al. 2009). Proportions of *P. gingivalis* in positive patients tend to increase progressively during pregnancy, with a peak at the third term and an abrupt decrease after delivery. The increase in *P. gingivalis* can be plausibly explained by the presence of progesterone in the medium, an overgrowth factor for this pathogen (Kornman & Loesche 1982). It should be borne in mind that classical studies grouped *P. gingivalis* together with *P. intermedia* such as black-pigmented *Bacteroides* (Jensen et al. 1981) before taxonomic reclassification (Shah & Collins 1988, 1990). In fact, there has been no previous report of an increase in *P. gingivalis* without grouping.

Although no significant change was found in *P. intermedia*, it also tended to increase during the pregnancy. In their review on subgingival microflora in pregnancy, Kornman & Loesche (1980) reported a significant increase in the percentage and the total colony-forming units of *P. intermedia* only during the second trimester, with a reduction during the third trimester and after delivery. If bacterial growth were mainly dependent on sex hormones, *P. intermedia* levels would show a greater increase and not a decrease during the third trimester (Mariotti 1994). Jensen et al. (1981) found a 55-fold increase in pregnant women and a 16-fold increase in women taking oral contraceptives, although their analysis was limited to black pigmented and *Fusobacterium* species in a cross-sectional study. Raber-Durlacher et al. (1994) performed a 14-day experimental gingivitis study after an intensive oral hygiene programme in the 25th week of pregnancy and repeated the model after delivery. In the pregnancy, the mean percentage of *P. intermedia* increased during experimental gingivitis (from 1.1% to 10%), but no such increase was observed post-partum. The same study detected no *P. gingivalis*, although plaque accumulation was only for 14 days and started after an intensive plaque control regimen, impeding colonization by *P. gingivalis*. Muramatsu & Takaesu (1994) also found a significant increase in *P. intermedia* during the second and the third trimester in a Japanese population.

A cross-sectional study (Jonsson et al. 1988) found no significant differences in the total counts and percentage of *P. intermedia* between pregnant and non-pregnant women or any correlation with

the progression of the pregnancy, but microbiological samples were only taken from seven patients in the second and the third trimesters. Adriaens et al. (2009) reported a significant decrease of *P. intermedia* levels between weeks 12 and 36 using a pairwise comparison, but an overgrowth of this pathogen was not stated. Gürsoy et al. (2009) observed a transient two times increase of *P. intermedia sensu lato* proportions during the second term, but differences were again not significant. On the other hand, this group corroborated our results of the significant overgrowth after delivery.

With regard to other sex-steroid gingivitis conditions, an increase in *P. intermedia* has also been described with the use of high-dose oral contraceptives (Klinger et al. 1998) and with the onset of puberty (Nakagawa et al. 1994). van Oosten et al. (1988) found an increase in black-pigmented *Bacteroides* and spirochaetes in pre-pubertal children. However, other authors found no differences (Gusberti et al. 1990).

Gingival inflammation in patients harbouring periodontal pathogens

A remarkable finding of the present study was the significantly greater gingival inflammation in the women who were positive for certain pathogens, suggesting that severe signs of gingival inflammation may develop in pregnant women who harbour specific pathogens (above culture threshold). *P. gingivalis* was associated with a significant increase in GI that was independent of the amount of plaque, whereas the increased inflammation observed in *P. intermedia*-positive women was concomitant with an increase in plaque levels. These results may explain, at least in part, why only some pregnant women develop more severe forms of gingivitis unrelated to the amount of plaque, due to the presence of certain periodontal pathogens, especially *P. gingivalis*. *P. gingivalis* and *P. nigrescens*, as well as *F. nucleatum* and *T. forsythia* have already been associated with bleeding on probing (Adriaens et al. 2009, Gürsoy et al. 2009).

The increase in *P. gingivalis* levels was correlated with a concomitant increase in the GI from the first to the second trimester, which strengthens the hypothesis that this pathogen plays a role in exacerbating inflammation. *P. intermedia* was also positively correlated with sex steroid hormones, in agreement with other reports (Kornman

& Loesche 1980, Muramatsu & Takaesu 1994, Nakagawa et al. 1994). Yokoyama et al. (2008) reported a correlation between oestradiol concentration and *C. rectus* levels that was not corroborated in our study, considering that the periodontopathogenic bacteria were determined in saliva samples.

Conclusion

Within the limitations of the present study, it can be concluded that pregnant women tended to present a more pathogenic bacterial profile compared with post-partum, which was concomitant with the worsening of clinical parameters. A more severe gingival inflammation was observed in pregnant women who harbour certain periodontal pathogens above the culture threshold from pregnancy onset, suggesting that the exacerbation of pregnancy gingivitis observed in some women may be, at least in part, related to a more pathogenic microbiological profile; however, more studies are needed for further investigation in this field.

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Address:
Ana Carrillo de Albornoz Sainz
Departamento de Estomatología III
Facultad de Odontología
Universidad Complutense de Madrid
Plaza Ramón y Cajal S/N
28040 Madrid
Spain
E-mail: anacarrillodea@gmail.com

Clinical Relevance

Scientific rationale for the study: The role of microbiological changes in the aetiopathogeny of pregnancy gingivitis has been widely researched,

but the published results have been controversial.

Principal findings: We found a qualitative change in subgingival bacteria composition concomitant with a worsening of gingival inflammation.

Practical implications: These findings contribute to our knowledge of the microbiology present throughout pregnancy, which is associated with the worsened gingival inflammation characteristic of this state.