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ORIGINAL ARTICLE

Gingival fluid cytokine expression and subgingival bacterial counts during pregnancy and postpartum: a case series

Regina Alessandri Bieri · Laurence Adriaens · Stefan Spörri · Niklaus P. Lang · G. Rutger Persson

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Abstract
Objectives The aim of this study was to assess gingival fluid (GCF) cytokine messenger RNA (mRNA) levels, subgingival bacteria, and clinical periodontal conditions during a normal pregnancy to postpartum.
Materials and methods Subgingival bacterial samples were analyzed with the checkerboard DNA–DNA hybridization method. GCF samples were assessed with real-time PCR including five proinflammatory cytokines and secretory leukocyte protease inhibitor.
Results Nineteen pregnant women with a mean age of 32 years (S.D. ± 4 years, range 26–42) participated in the study. Full-mouth bleeding scores (BOP) decreased from an average of 41.2% (S.D. ± 18.6%) at the 12th week of pregnancy to 26.6% (S.D. ± 14.4%) at the 4–6 weeks postpartum ($p<0.001$). Between week 12 and 4–6 weeks postpartum, the mean probing pocket depth changed from 2.4 mm (S.D. ± 0.4) to 2.3 mm (S.D. ± 0.3) ($p=0.34$). Higher counts of *Eubacterium saburreum*, *Parvimonas micra*, *Selenomonas noxia*, and *Staphylococcus aureus* were found at week 12 of pregnancy than at the 4–6 weeks postpartum examinations ($p<0.001$). During and after pregnancy, statistically significant correlations between BOP scores and bacterial counts were observed. BOP scores and GCF levels of selected cytokines were not related to each other and no differences in GCF levels of the cytokines were observed between samples from the 12th week of pregnancy to 4–6 weeks postpartum. Decreasing postpartum counts of *Porphyromonas endodontalis* and *Pseudomonas aeruginosa* were associated with decreasing levels of Il-8 and Il-1β.
Conclusions BOP decreased after pregnancy without any active periodontal therapy. Associations between bacterial counts and cytokine levels varied greatly in pregnant women with gingivitis and a normal pregnancy outcome. Postpartum associations between GCF cytokines and bacterial counts were more consistent.
Clinical relevance Combined assessments of gingival fluid cytokines and subgingival bacteria may provide important information on host response.

Keywords Pregnancy · Bacteria · Cytokine · Gingival inflammation · Gingival crevicular fluid

R. A. Bieri · L. Adriaens · G. R. Persson (✉)
Department of Periodontology, University of Bern,
Freiburgstrasse 7,
CH-3010 Bern, Switzerland
e-mail: rutger.persson@zmk.unibe.ch

S. Spörri
Department of Obstetrics and Gynecology,
State Hospital of Fribourg,
Fribourg, Switzerland

N. P. Lang
Faculty of Dentistry, Prince Philip Dental Hospital,
The University of Hong Kong,
Hong Kong, SAR, People's Republic of China

G. R. Persson
Department of Periodontics and Department of Oral Medicine,
University of Washington,
Seattle WA, USA

Introduction

During pregnancy, gingival inflammation is common. Pregnancy gingivitis usually resolves spontaneously within a few weeks after delivery [1–3]. Data suggest that temporary clinical periodontal changes during pregnancy do not result in an increased risk for future periodontitis [4]. During the second trimester of pregnancy, a shift of the subgingival

levels of *Prevotella intermedia* and *Porphyromonas gingivalis* has been observed [2, 5, 6]. Other authors have not found any differences in the composition of the oral biofilm between pregnant and nonpregnant women [4, 7]. Why within a few weeks after delivery, increased gingival inflammation during pregnancy is quickly resolved, remains unclear. In a previous publication including the same subjects as in the present work, we identified that bacterial counts of bacteria associated with periodontitis did not change during pregnancy or to a time point at 4–6 weeks postpartum [8].

Recent data suggest that cytokine levels in saliva may be linked to the periodontal status [9]. Other studies have not been able to demonstrate that salivary levels of granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) can discriminate between periodontal health and disease [10].

Studies of cytokine levels in gingival crevicular fluid (GCF) have suggested that TNF-α levels are lower at healthy sites in periodontally healthy subjects than in subjects with advanced periodontitis, who have high levels of TNF-α in GCF both at sites with or without evidence of disease [11]. The influence of sex hormones on proinflammatory cytokines in GCF of healthy premenopausal, nonpregnant women has recently been studied. No associations between clinical periodontal parameters, hormonal changes, and differences in GCF cytokine levels were found [12]. In a study with pregnant women, exacerbated gingival inflammation during pregnancy could not be associated with increased salivary progesterone or estradiol levels or increased levels of proinflammatory cytokines [13].

Secretory leukocyte protease inhibitor (SLPI) is present in human fluids and also in gingival tissues [14]. SLPI is a common antimicrobial protein of the upper airways [15] inhibiting LPS and nuclear factor-κB (NF-κB) activation [16]. SLPI exhibiting strong antibacterial activities has been identified in GCF [17]. Furthermore, SLPI causes an upregulation of the production of anti-inflammatory cytokines [18]. In vitro studies have, however, also recently demonstrated that arginine-specific gingipains of *P. gingivalis* inhibit the protective effects of SLPI [14]. Currently, it is unknown if GCF levels of SLPI change during pregnancy, or if the presence or absence of oral bacteria influences the GCF levels of SLPI in pregnancy.

Up to date, we have only limited information about the relationship between bacteria in periodontal pockets/sulci and the presence or absence of proinflammatory cytokines in pregnant women. Therefore, the purposes of the present study were to assess:

1. Microbiological changes between week 12 of pregnancy to 4–6 weeks postpartum and in relation to changes in gingival inflammation (percent sites with bleeding on probing (BOP)) between week 12 of pregnancy and 4–6 weeks postpartum
2. The association between levels of mRNA of proinflammatory cytokines in gingival fluid and the severity of gingival inflammation (percent sites with BOP) at week 12 of pregnancy and 4–6 weeks postpartum
3. Differences in GCF cytokine levels of mRNA (IL-1α, IL-1β, IL-8, TNF-α, and SLPI) at the 12th week of pregnancy and 4–6 weeks postpartum
4. The association between changes in bacterial and cytokine levels between week 12 of pregnancy and 4–6 weeks postpartum

Materials and methods

The Institutional Review Board of the Cantons of Jura, Fribourg, and Neuchâtel, Switzerland approved the present study. All the participating subjects signed informed consent. The study was a prospective consecutive case series over the period of pregnancy (from week 12 through 4–6 weeks postpartum) without study-defined medical or dental interventions.

Subject population

Pregnant women, 18 years of age or older, attending the Department of Obstetrics and Gynecology at the State Hospital of Fribourg, Switzerland and with singleton pregnancy were included. Women with known congenital uterine malformations, congenital vaginal malformations, fetal malformation, multifetal gestation, and with chronic diseases (e.g., diabetes, hypertension, epilepsy, cardiac disease, lung disease, renal disease, and positive test for human immunodeficiency virus (HIV)) were excluded. All the participating women were monitored by standard of care principles at the Department of Obstetrics and Gynecology. The subjects were recruited between April and October 2006. None of the women had received periodontal therapy before and during pregnancy.

GCF sampling and processing

GCF was collected with sterile endodontic paper points (absorbent paper points, Dentsply/Maillefer, Ballaigues, Switzerland), and parallel to this procedure, subgingival bacterial samples were also collected using sterile endodontic paper points. GCF and subgingival bacterial samples were harbored from the mesiobuccal aspects of all first molars, and each paper point remained in situ for 15 s. The first molars were chosen while these teeth are

commonly present in young adults and evidence of inflammation at these teeth are common. In addition, the first molars are simple to isolate and easy to collect samples from. The samples were placed in separate Eppendorf tubes (1.5 ml natural flat cap microcentrifuge tubes, Starlab, Ahrensburg, Germany) for the assessment of cytokines and bacteria. All the samples were immediately transported on dry ice to the Oral Microbiology Laboratory of the University of Bern, Switzerland for processing.

Clinical examination

A full-mouth periodontal examination with the assessments of BOP and probing pocket depth (PPD) was performed at the 12th week of pregnancy and at a visit 4–6 weeks after delivery. BOP, PPD, and gingival recession were measured at six sites per tooth using the Florida probe® system (Florida Probe Corporation, Gainesville, FL, USA) with a standardized pressure of 0.15 N. The same examiner (L. A.) performed the periodontal measurements. Gingivitis was defined as having >20% of sites with BOP if periodontitis was not found. Periodontitis was defined as having periodontal sites with probing pocket depths >4 mm and with radiographic evidence of alveolar bone loss as defined from post pregnancy intraoral radiographs.

Analysis of subgingival bacterial samples

The bacterial samples were stored at −20°C and processed after a standard storage period of 3 months. To each sample, 0.15 ml TE (10 mM Tris–HCL, 1 mM EDTA, pH 7.6) and 0.5 ml NaOH were added. All the bacterial samples were analyzed with the checkerboard DNA–DNA hybridization technique. The 74 species assessed are presented in Table 1. The checkerboard DNA–DNA hybridization was performed as described elsewhere [19, 20]. Briefly, bacterial DNA was extracted using the method of Smith et al. [21], concentrated on nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany), and fixed by cross-linking using ultraviolet light (Stratalinker 1800, Stratagene, La Jolla, CA, USA). The membranes with fixed DNA were placed in a Miniblotter 45 (Immunitics, Cambridge, MA, USA). Signals were detected by fluorescence using the Storm Fluor-Imager (Storm 840, Amersham Biosciences, Piscataway, NJ, USA) with a setup of 200 μm and 600 V. The digitized information was analyzed by a software program (ImageQuant, Amersham Pharmacia, Piscataway NJ, USA), allowing comparison of the density of the 19 sample lanes against the 2 standard lanes (10^5 or 10^6 cells). Signals were converted to absolute counts by comparisons with these standards [19]. Subject-based mean values for bacterial counts of these species were calculated and used in the assessments.

GCF analysis of cytokines

RNA extraction

The GCF samples were frozen at −79°C. After thawing, the lysis buffer (RNAqueous®-Micro kit, Ambion, Austin, TX, USA) was added to the Eppendorf tube for 10 min. EtOH 100% was added, and the mixture was loaded on the Micro Filter Cartridge assembly (RNAqueous®-Micro kit, Ambion, Austin, TX, USA). Further processing was performed according to manufacturer's instructions. The eluted RNA was treated with DNAse 1 (RNAqueous®-Micro kit, Ambion, Austin, TX, USA).

Reverse transcription and quantitative real-time polymerase chain reaction

The total RNA used for quantitative real-time polymerase chain reaction (QRT-PCR) was reversely transcribed using random primers and MultiScribe™ reverse transcriptase according to manufacturer's instructions (high-capacity cDNA reverse transcription kit, Applied Biosystems, Foster City, CA, USA). Transcribed reactions were analyzed in real-time on an ABI 7500 (Applied Bio Systems, Foster City, CA, USA) using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Primers and probes for detection of cytokines were all purchased from Applied Biosystems. Thermal cycling parameters were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Reactions were performed in triplicates and threshold cycle numbers were averaged. The threshold data generated were normalized to GAPDH and UBB, calculating Δct. These housekeeping genes were chosen after analyzing 10 different endogenous controls with 12 GCF samples. The amount of cDNA was standardized for all reactions. The samples were collected from 12 different randomly selected nonpregnant individuals and therefore not part of the study (data not shown).

Statistical methods

Statistical analysis was performed using Wilcoxon signed rank test for within subject changes over time. Mann–Whitney *U* tests were also used to assess changes over time. Correlation coefficients between parameters were assessed by Pearson correlation coefficient. Analysis by linear (stepwise) regression was performed to identify explanatory models to GCF cytokine changes during pregnancy.

Table 1 Bacteria included in the checkerboard DNA–DNA hybridization assays

Bacteria	Collection	Bacteria	Collection
Aggregatibacter actinomycetemcomitans (a)	ATCC29523	*Actinomyces neuii*	GUH550898
Aggregatibacter actinomycetemcomitans (Y)	ATCC43718	*Aerococcus christensenii*	GUH070938
Actinomyces israelii	ATCC 1201	*Anaerococcus vaginalis*	GUH290486
Actinomyces naeslundii	ATCC121045	*Atopobium parvulum*	GUH160323
Actinomyces odontolyticus	ATCC17929	*Atopobium vaginae*	GUH010535
Capnocytophaga gingivalis	ATCC33612	*Bacteroides ureolyticus*	GUH080189
Capnocytophaga ochracea	ATCC33596	*Bifidobacterium biavatii*	GUH071026
Capnocytophaga sputigena	ATCC33612	*Bifidobacterium bifidum*	GUH070962
Campylobacter gracilis	ATCC33236	*Bifidobacterium breve*	GUH080484
Campylobacter rectus	ATCC33238	*Bifidobacterium longum*	GUH180689
Campylobacter showae	ATCC451146	*Corynebacterium nigricans*	GUH450453
Eikenella corrodens	ATCC238345	*Corynebacterium aurimucosum*	GUH071035
Eubacterium saburreum	ASTCC33271	*Dialister sp.*	GUH071035
Fusobacterium nucl. naviforme	ASTCC49256	*Enterococcus faecalis*	GUH170812
Fusobacterium.nucl.nucleatum	ATCC25586	*Enterococcus faecalis*	ATCC29212
Fusobacterium.nucl. polymorphum	ATCC10953	*Echerichia coli*	GUH070903
Fusobacterium periodonticum	ATCC33993	*Gardnerella vaginalis*	GUH080585
Lactobacillus acidophilus	ATCC11975	*Haemophilus influenzae*	ATCC49247
Leptothrichia buccalis	ATCC14201	*Helicobacter pylori*	ATCC43504
Neisseria mucosa	ATCC33270	*Lactobacillus crispatus*	GUH160342
Parvimonas micra	ATCC19696	*Lactobacillus gasseri*	GUH170856
Prevotella intermedia	ATCC25611	*Lactobacillus iners*	GUH160334
Prevotella melaninogenica	ATCC25845	*Lactobacillus jensenii*	GUH160339
Prevotella nigrescens	ATCC33563	*Lactobacillus vaginalis*	GUH0780928
Porphyromonas gingivalis	ATCC33277	*Mobiluncus curtisii*	GUH070927
Propionybacterium acnes	ATCC11827/28	*Mobiluncus mulieris*	GUH070926
Selenomonas noxia	ATCC43541	*Peptoniphilus sp.*	GUH550970
Streptorcoccus anginosus	ATCC33397	*Peptostreptococcus anaerobius*	GUH160362
Streptococcus constellatus	ATCC27823	*Porphyromonas endodontalis*	ATCC35406
Streptococcus gordonii	ATCC10558	*Prevotella bivia*	GUH450429
Streptococcus intermedius	ATCC27335	*Prevotella disiens*	GUH190184
Streptococcus mitis	ATCC49456	*Proteus mirabilis*	GUH070918
Streptococcus mutans	ATCC25175	*Pseudomomas aeruginosa*	ATCC33467
Streptococcus oralis	ATCC35037	*Staphylococcus aureus*	ATCC25923
Streptococcus sanguinis	ATCC10556	*Staphylococcus.aureus yellow strain*	GUH070921
Tannerella forsythia	ATCC43037	*Staphylococcus aureus white strain*	GUH070922
Treponema denticola	ATCC354405	*Staphylococcus epidermis*	DSMZ20044
Treponema socranskii	D40DR2	*Staphylococcus haemolyticus*	DSMZ20263
Veillonella parvula	ATCC10790	*Streptococcus agalactiae*	GUH230282
		Varibaculum cambriense	GUH070917

ATCC American type culture collection, *D* sample from Forsyth Institute, Boston, MA, USA, *GUH* Ghent University Hospital Collection, Ghent, Belgium

Results

The mean age of the 19 women, who all delivered a child without complications, was 32 years (S.D. ± 4 years, range 26–42). Clinical periodontal conditions during the pregnancies have been reported elsewhere [8]. Briefly, and at the 12th week of pregnancy, 1.5% of sites (six sites per tooth examined) had a PPD >4 mm which changed to 1.4% at the

4–6 weeks postpartum examination. At week 12, the mean PPD was 2.4 mm (S.D. ± 0.4). At 4–6 weeks postpartum, the mean PPD was 2.3 mm (S.D. ± 0.3) (p=0.34). Evidence of gingival hyperplasia was found at 8.7% of sites at the 12th week of pregnancy and at 9.1% at 4–6 weeks postpartum. On average, these women had 29 teeth (S.D. ± 1.5). A clinical diagnosis of gingivitis was made in all of the 19 subjects. None of the subjects could be identified as having periodontitis. A decrease in BOP scores from a mean of 41.2% (S.D. ± 18.6%) at week 12 of pregnancy to 26.6% (S.D. ± 14.4%) at the 4–6 weeks postpartum examination was found (p<0.001). The proportion of sites with BOP at week 12 was significantly correlated with the proportion of sites with BOP at 4–6 weeks postpartum (r=0.73, p<0.001) (Fig. 1).

Microbiological changes between week 12 of pregnancy to 4–6 weeks postpartum and in relation to changes in gingival inflammation (in percent, sites with BOP) between week 12 of pregnancy and 4–6 weeks postpartum

The distributions of selected bacteria are presented (Table 2). At week 12, as well as at the 4–6 weeks postpartum examinations, no differences in bacterial counts were found between women with a diagnosis of gingivitis or periodontitis, respectively. Thus, independent of the periodontal diagnosis, the following microorganisms were found at significantly higher counts at week 12 than at the microbiological sampling 4–6 weeks postpartum: *Eubacterium saburreum*, *Parvimonas micra*, *Selenomonas noxia*, and *Staphylococcus aureus* (p<0.001). *Mobiluncus curtisii* presented with the opposite pattern. The changes for *S. aureus* and *P. micra* are presented in Fig. 2.

Table 2 Frequency distribution (in percent) of selected bacteria at week 12 and at 4–6 weeks postpartum at 10^4 and 10^5 bacterial cell cutoff levels

	Week 12		4-6 weeks post-partum	
	10^4 cells	10^5 cells	10^4 cells	10^5 cells
E. saburreum	100.0	5.3	100.0	0.0
M. curtisii	5.3	0.0	43.1	9.0
P. micra	100.0	5.3	84.0	0.0
S. noxia	100.0	10.5	73.7	5.3
S. aureus	100.0	36.8	89.5	5.3

The subject-based proportions of BOP scores at week 12 of pregnancy were significantly correlated with the bacterial counts of *Actinomyces israelii* (r=0.62, p=0.006), *Actinomyces naeslundii* (r=0.56, p=0.012), *Actinomyces odontolyticus* (r=0.61, p=0.006), *Bifidobacterium bifidum* (r=0.61, p=0.006), *Lactobacillus acidophilus* (r=0.60, p=0.006), *Treponema socranskii* (r=0.61, p=0.006), and *P. gingivalis* (r=0.56, p=0.012).

At 4–6 weeks postpartum, statistically significant correlations between the BOP scores and the bacterial counts were found for: *Actinomyces neuii* (r=0.57, p<0.01), *Atopobium parvulum* (r=0.61, p=0.006), *Bifidobacterium biavatii* (r=0.61, p=0.006), *B. bifidum* (r=0.71, p=0.001), *Bifidobacterium breve* (r=0.71, p=0.001), *Corynebacterium aurimucosum* (r=0.70, p=0.001), *Corynebacterium nigricans* (r=0.63, p=0.001), *Enterococcus faecalis* (r=0.63, p=0.003), *M.*

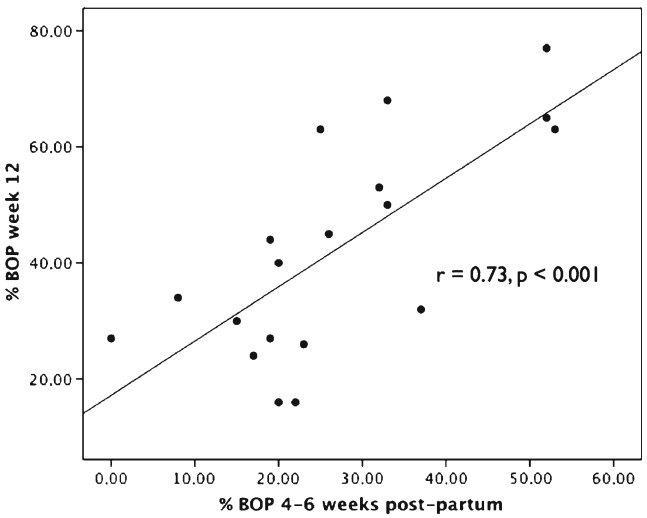

Fig. 1 Scatterplot diagram illustrating the relationship between percent BOP scores at week 12 of pregnancy and percent BOP scores at 4–6 weeks postpartum

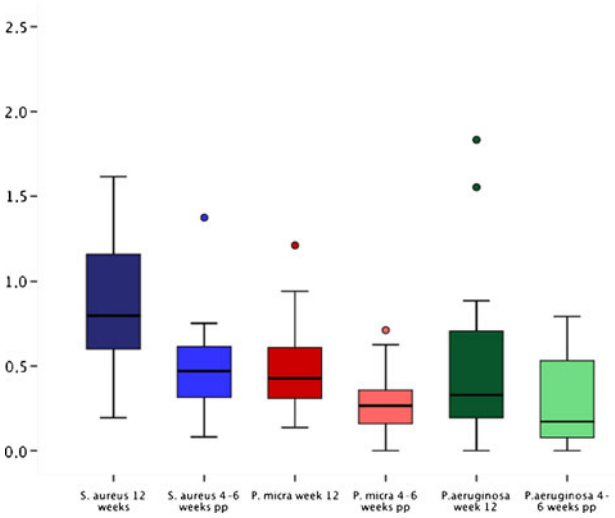

Fig. 2 Boxplot diagram illustrating the levels of *S. aureus* and *P. micra* at week 12 of pregnancy and 4–6 weeks postpartum. *X*-axis= 1.0×10^5 bacterial cells; *circles with different colors* outlier values

curtisii (r=0.68, p=0.001), *Mobiluncus mulieris* (r=0.71, p=0.001), *P. intermedia* (r=0.62, p=0.005), *Pseudomonas aeruginosa* (r=0.002, p=0.001), *Tannerella forsythia* (r=0.59, p=0.008), *Streptococcus agalactiae* (r=0.58, p=0.007), *Treponema denticola* (r=0.89, p=0.001), and *T. socranskii* (r=0.61, p=0.006).

The significant correlations between changes in bacterial levels are presented (Table 3). The highest level of a correlation in bacterial counts between week 12 of pregnancy and 4–6 weeks postpartum was identified between changes of *Porphyromonas endodontalis* and *P. aeruginosa* (r=0.99, p<0.001).

Association between levels of mRNA of proinflammatory cytokines in gingival fluid and the severity of gingival inflammation (in percent, sites with BOP) at week 12 of pregnancy and 4–6 weeks postpartum

Neither at the week 12, nor at the 4–6 weeks post-partum examinations were BOP scores correlated with cytokine values.

Differences in GCF cytokine levels of mRNA (IL-1α, IL-1β, IL-8, TNF-α, and SLPI) at the 12th week of pregnancy and 4–6 weeks postpartum

At week 12, as well as at the 4–6 weeks postpartum examination, no differences in the GCF mRNA cytokine levels studied were found between women with a diagnosis of gingivitis or periodontitis. Thus, with all cases, included statistical analysis failed to demonstrate differences in GCF levels of mRNA: IL-1α, IL-1β, IL-8, TNF-α, and SLPI between samples from the 12th week of pregnancy and 4–6 weeks postpartum.

GCF levels of mRNA IL-8 were significantly correlated with mRNA IL-1β levels (r=0.80, p<0.001). GCF levels of mRNA IL-8 were also correlated with mRNA TNF-α levels (r=0.63, p=0.003).

The GCF levels of mRNA SLPI at both the 12th week of pregnancy and at the 4–6 weeks postpartum assessments were significantly correlated with GCF levels of MRNA IL-1α (r=0.49, p<0.001), (r=0.69, p=0.001), respectively. The reverse relationship between the changes in mRNA SLPI and mRNA Il-1α between week 12 and 4–6 weeks postpartum (r=−0.71, p<0.01) is presented in a scatterplot diagram (Fig. 3). GCF levels of mRNA IL-8 and mRNA IL-1β were correlated with mRNA TNF-α levels (r=0.66, p=0.002) and (r=0.66, p<0.002) and correlated with the GCF levels of mRNA IL-1α (r=−0.71, p<0.001), respectively. The changes in the levels of mRNA IL-8 and mRNA IL-1β between week 12 of pregnancy and the 4–6 week postpartum assessments were also significantly correlated (r=−0.88, p<0.001). Statistical analysis failed to demonstrate correlations between changes in levels of the other cytokine combinations. Data on the mRNA TNF-α levels, mRNA Il-8, mRNA Il-1β, and SLPI at week 12 and 4–6 weeks postpartum are presented in a boxplot diagram (Fig. 4).

The association between changes in bacterial and cytokine levels between week 12 of pregnancy and 4–6 weeks postpartum

Statistically significant correlations between changes in bacterial counts at 12 weeks of pregnancy and postpartum are presented (Table 4). Decreasing levels of *P. endodontalis* and *P. aeruginosa* were correlated with decreasing levels of mRNA Il-8 and mRNA Il-1β. Decreasing levels of *Bacteroides ureolyticus* were correlated with decreasing expression of mRNA Il-1α (r=0.48, p<0.05). It was noticeable that no correlations in changes in cytokine expression and bacterial changes were found for bacteria associated with periodontitis (*P. gingivalis, T. forsythia,* and *T. denticola*).

Table 3 Significant correlations between changes in subgingival counts of bacteria between week 12 of pregnancy and 4–6 weeks postpartum

	A. neuii	*A. parvula*	*B. biavatii*	*E. faecalis*	*M. curtisii*	*P. aeruginosa*	*P. micra*	*P. endodontalis*	*S. aureus*	*S. agalactiae*
A. neuii					0.61***	0.62***		0.63***		
A. parvula			0.85***	0.83***	0.52*					0.81***
B. biavatii		0.95***			0.56**					0.87***
E. faecalis		0.83***			0.46*					0.85***
M. curtisii	0.61***	0.52*	0.56**	0.46*		0.73***		0.71***		
P. aeruginosa	0.62***				0.73***			0.99***		0.64***
P. micra									0.67***	
P. endodontalis	0.63***				0.71***	0.99***				0.60**
S. aureus							0.67***			
S. agalactiae		0.81***	0.87***	0.85***		0.64***		0.60**		

*p<0.05; **p<0.01; ***p<0.001

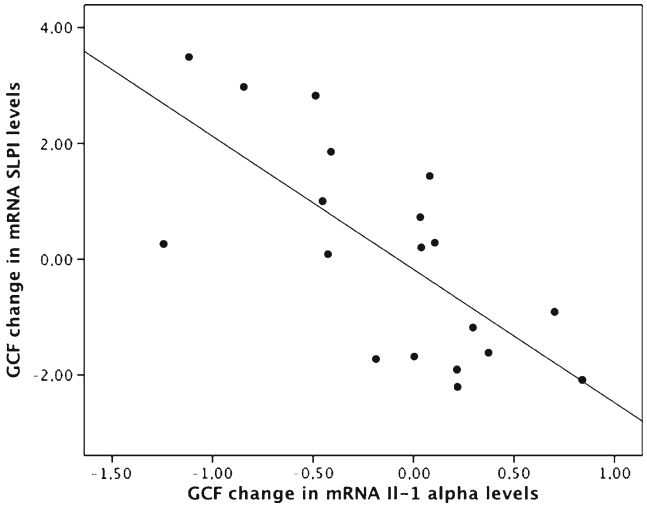

Fig. 3 Scatterplot diagram illustrating the relationship between changes in mRNA SLPI and mRNA IL-1α between week 12 of pregnancy and the 4–6 weeks postpartum analysis

Explanatory variables to cytokine levels at week 12 and 4–6 weeks postpartum

GCF levels of mRNA IL-8 and mRNA IL-1β were explained by bacterial counts of *P. endodontalis* (t=3.4, p<0.05), (t=4.6, p<0.001), respectively. GCF levels of mRNA IL-1α were explained by bacterial counts of *B. ureolyticus* (t=5.7, p<0.001). High GCF levels of mRNA SLPI were associated with low counts of *P. aeruginosa* (t=−3.1, p<0.001). GCF

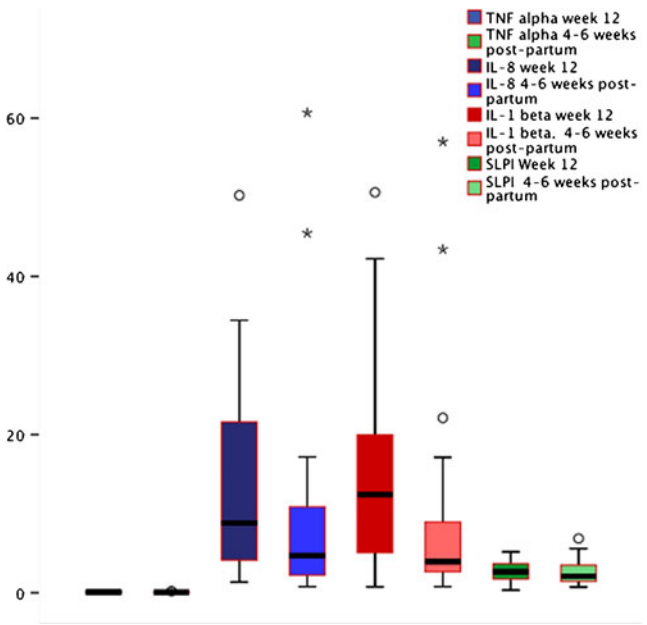

Fig. 4 Boxplot diagram illustrating mRNA cytokine levels at week 12 and at 4–6 weeks postpartum (*empty circles* represent outlier values and *asterisks* represents extreme outliers. *Whiskers* represent 25th and 75th percentiles)

Table 4 Statistically significant correlation coefficients between changes in cytokine and bacterial counts

	IL-1α	IL-1β	IL-8	TNF-α
B. ureolyticus	−0.48*			
C. aurimucosum				0.47*
M. curtisii		−0.51*	−0.57**	
M. mulieris		−0.61***	−0.69***	
P. aeruginosa		−0.47**	−0.54*	
P. endodontalis		−0.52*	−0.58*	
S. agalactiae			−0.50*	

*p<0.05; **p<0.01; ***p<0.001 (Pearson's correlation)

levels of mRNA TNF-α could not be explained by any of the counts of microorganisms studied.

Discussion

In the present study, pregnant women with a normal pregnancy outcome consecutively admitted to an obstetrics and gynecology ward were examined. The subject sample size in the present study was small but comparable to several other studies that have assessed specific factors in relation to pregnancy and periodontal conditions. One of the difficulties in assessing periodontal status during pregnancy can be the absence of radiographic evidence of bone loss or the interpretation of bone levels. Another problem may be the rather common finding of gingival hyperplasia that was found in 9% of the subjects in the present study. When we applied the same criteria as those used by Novak et al. 2008 [22] to the subjects in the present study all the pregnant women were diagnosed as only having gingivitis at both the 12th week and at the postpartum examination. The alveolar bone levels as assessed from available radiographs were within normal limits as was expected in this age cohort and clinical presentation.

Others have reported that approximately 40% to 50% of pregnant women have gingivitis [23]. In the present study population, the mean extent of BOP was 41.2% at 12 weeks of pregnancy. Although all subjects with no influence by the investigators had abstained from professional dental care during the pregnancy, the BOP scores decreased to, on average, 26.6% at 4–6 weeks postpartum. The decrease in the extent of gingival inflammation could be the result of a physiological change, which may be explained by hormonal changes during pregnancy [2, 24]. This would then also suggest that the hormonal changes may have an impact on mRNA cytokine expression in gingival tissues and fluids, while maintaining a balanced bacterial biofilm. In a recent study, data suggested that in premenopausal women only GCF levels of mRNA IL-6 levels peaked at the time of

ovulation. This was not accompanied by clinical changes in the extent of gingival inflammation [25]. These authors failed to show changes in GCF levels of other proinflammatory cytokines during a normal menstrual cycle. Thus the impact of hormonal changes on gingival inflammation is unclear. In the present study, the change in mean PPD between week 12 and 4–6 weeks postpartum was statistically not significant and also clinically irrelevant. This may, in part explain the results obtained in regards to GCF changes in cytokines and bacterial counts.

The scientific evidence in regard to the subgingival microbiota during pregnancy in regard to bacterial counts is inconsistent [2, 4–7]. This may, in part, be the result of differences in sampling methods and analysis, diagnostic criteria, treatment provided or not, age of the pregnant subjects, general health status, and, i.e., smoking habits. The observations in the present study suggested that the microbiota assessed overall was stable between the 12th week of pregnancy and until 4–6 weeks postpartum some species would suggest that hormonal changes may not result in microbiological shifts.

The present study also identified that the extent of BOP both at week 12 of pregnancy and at 4–6 weeks postpartum was associated with a complex microbiota dominated by bacteria associated with gingivitis. Significantly higher counts of *E. saburreum*, *P. micra*, *S. noxia*, and *S. aureus* were, however, found at week 12 of pregnancy than at the 4–6 week postpartum examination, suggesting a decrease for these species. In contrast *M. curtisii* increased from week 12 of pregnancy to 4–6 week postpartum. *E. saburreum*, *M. curtisii*, and *S. noxia* are commensal oral bacteria, while *P. micra* is a gram-positive, anaerobic bacterium and has been associated with periodontal destruction [26]. *S. aureus* infection has been associated with periodontitis [27] but is also found in periodontally healthy subjects. It is noticeable that the counts of bacteria commonly associated with periodontitis did not change during the study period. The overall stable oral microbiota noticed in the present study is consistent with the perception of a stable vaginal microbiota during normal pregnancies and in the absence of bacterial vaginosis.

The inclusion of a study method to assess mRNA cytokine levels in GCF was made based on a common principle to assess various enzymes in GCF and primarily expressed by immune competent cells, i.e., polymorphonuclear cells (PMN cells). PMN cells constitute a major component of the cellular component in GCF.

No differences in mRNA detected in GCF for IL-1α, IL-1β, IL-8, TNF-α, and SLPI between week 12 and postpartum were found. Thus, the changes in gingival inflammation (BOP) may only weakly be associated with the expression of these selected cytokines in GCF during pregnancy.

In the present study, consistent correlations between the levels of mRNA: IL-1β, TNF-α, and IL-8 during pregnancy and postpartum were identified. IL-1β and TNF-α are potent proinflammatory cytokines. IL-1β induces chemotaxis of leukocytes by inducing the production of IL-8 [28]. During pregnancy, there is a decrease in B cell response due to progressive increase of progesterone and estrogens, which reaches the peak in the third trimester [29]. *P. endodontalis* LPS is capable of stimulating PMNs to produce chemotactic cytokines [30]. In the present study, an upregulation of IL-8 and IL-1β was associated with higher levels of *P. endodontalis*.

Information about the relationship between bacteria in periodontal pockets/sulci and presence or absence of proinflammatory cytokines is limited. This may partly be explained by difficulties to accurately assess levels of proinflammatory cytokines in GCF. The use of a high-capacity cDNA reverse transcription method using housekeeping genes as standard references and to use gene expression to quantify cytokines [31, 32] in relation periodontal inflammation and bacterial presence during pregnancy might overcome this problem.

SLPI is a neutrophil elastase inhibitor with antibacterial and with anti-inflammatory properties [33]. While SLPI is associated with antibacterial and anti-inflammatory conditions, IL-1α suggests an early phase of inflammation. In the present study a significant correlation was found between the changes in GCF levels of SLPI and IL-1α both at week 12 of pregnancy and postpartum. The period between the 12th week of pregnancy and the postpartum examination may therefore reflect a transient phase of inflammation in pregnant/post-delivery women.

In the present study, the levels of the SLPI cytokine were inversely correlated with counts of *P. aeruginosa* but only postpartum. *P. aeruginosa* is a known pathogen in chronic infections and identified in periodontitis [34] High counts of SLPI are consistent with low counts of pathogens, which is consistent with periodontal conditions postpartum in the women participating in the present study.

In gingival inflammation, GCF is secreted by and through the sulcular epithelium and this exudate is rich in cellular elements specifically leucocytes, primarily neutrophils, and lymphocytes. Bacterial stimuli induce production of proinflammatory cytokines facilitating the host immune system to control the infectious agents. Data suggest that the levels of cytokines in GCF are associated with periodontal disease status [35]. However bacteria, i.e., *P. gingivalis* can modulate important host signaling cytokine responses [36]. In the present study, we assessed mRNA expression of proinflammatory cytokines and the SLIPI while accounting for bacterial presence in GCF. The lack of agreement between bacterial levels in GCF and the assessments of cytokine messenger mRNA identified in the present study may reflect the imbalance in the abilities between host and infectious agents which was also reflected by gingival status

in pregnant women. It might be that the primary line of host defense is not in GCF but in gingival tissues and where the mRNA expression of cytokines through competent immune cells prevent bacteria invasion.

To conclude, BOP decreased between the week 12 of pregnancy and postpartum. Therefore, moderate gingivitis during pregnancy may partially resolve without any active therapy. GCF levels of proinflammatory cytokines were correlated with each other. Specifically decreasing expression of proinflammatory cytokines and levels of some bacteria (i.e., *B. ureloyticus, P. aeruginosa*, and *P. endodontalis*) appears to be explanatory to clinical periodontal improvements 4–6 weeks postpartum. However, we were unable to explain the difference in susceptibility to pregnancy gingivitis with elevated proinflammatory cytokine levels during pregnancy. In the future, studies with larger populations are needed in this field of research.

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