

SALIVARY ANTIMICROBIAL DEFENSINS IN PREGNANCY

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Abstract

Aim: Susceptibility to and severity of gingival inflammation are enhanced during pregnancy; however, regulation of oral innate-immune response, including antimicrobial peptides, during pregnancy is still unknown. We analyzed salivary levels of human beta-defensin (hBD)-1, -2, -3, and human neutrophilic peptide (HNP)-1 in pregnant women, and related those to their periodontal status.

Material and Methods: In this cohort study, 30 generally healthy, non-smoking Caucasian women without periodontitis were followed at three time-points during pregnancy and twice post-partum. The non-pregnant group consisted of 24 women, who were examined three times at the following months. At each visit, periodontal status was recorded and stimulated saliva samples were collected. Salivary estradiol, progesterone, and defensin concentrations were measured by ELISA assays.

Results: After adjusting for visible plaque and gingival bleeding, reduced salivary concentrations of hBD-1, hBD-2, and HNP-1 were found especially during the third trimester, whereas hBD-3 concentrations did not change during pregnancy and post-partum visits. Weak associations were observed between salivary defensin and hormone concentrations and clinical parameters.

Conclusion: There seems to be an independent regulation cascade for each antimicrobial defensin in the oral cavity during pregnancy, despite of the similarities between these antimicrobial peptides.

Clinical Relevance:

Scientific rationale for study: Relationship between salivary antimicrobial defensins and periodontal status was examined during pregnancy and post-partum and then compared with matched non-pregnant women.

Principal findings: After adjustments to clinical parameters, human beta-defensin (hBD)-1, -2, and human neutrophilic peptide-1 concentrations in saliva decreased during the third trimester and increased after delivery, while concentrations of hBD-3 remained stable during the follow-up.

Practical implications: Salivary hBD-3 can be one important protective agent of the oral cavity in gestation, because it is not affected by pregnancy-related changes like other salivary defensins.

Introduction

Human beta-defensins (hBDs) and human alpha-defensins (human neutrophilic peptides, HNPs) are a group of cationic antimicrobial peptides that take part in the innate host response against microbes. In the oral cavity, hBDs are produced by the epithelium (Yin et al. 2010; Greer et al. 2013), while HNPs are mainly expressed in the azurophilic granules of polymorphonuclear neutrophils (PMNs) (Rice et al. 1987). Both epithelial and neutrophilic peptides demonstrate broad-spectrum antimicrobial effects against Gram-positive and Gram-negative bacteria (Ganz et al. 1985, Garcia et al. 2001, Dommisch et al. 2012), viruses (Daher et al. 1986, Quiñones-Mateu et al. 2003), and fungi (Garcia et al. 2001, Feng et al. 2005). Antimicrobial killing by defensins include several different mechanisms, such as forming pores on outer membranes of bacteria, as well as triggering lysis and leakage of intracellular proteins. Moreover, epithelial defensins take part in immune response as being chemotactic to T cells, while neutrophilic peptides regulate the homeostasis of gingival epithelium through their effects on epithelial cell attachment, spread, and proliferation (Greer et al. 2013, Gursoy & Könönen 2012, Gursoy et al. 2013).

Pregnancy is a unique immune state, where the maternal immune system is re-modulated to protect the mother against environmental challenges, but is also programmed of preventing to reject the fetus. Gestation includes three distinct immunological phases; proinflammatory first trimester, anti-inflammatory second trimester, and proinflammatory third trimester (Mor & Cardenas 2010). Establishment and maintenance of pregnancy are regulated by steroid hormones (i.e., estradiol, progesterone, and gonadotropin). These hormones regulate the recruitment and functions of dendritic cells, lymphocytes, macrophages, and essentially neutrophils (Schumacher et al. 2014). PMNs take part in innate immune response against infection, and they participate in the process of delivery by releasing proinflammatory cytokines and secreting matrix metalloproteinases (MMPs)

(Gomez-Lopez et al. 2014). Another group of innate response proteins, epithelial hBDs, are widely expressed in the amnion, deciduas, placenta, and chorion during gestation (Stock 2007, Frew & Stock 2011). Clinical changes in the periodontal environment during pregnancy are characterized by an increased tendency to gingival bleeding (Gürsoy et al. 2008, Figuero et al. 2013). Despite the elevated inflammatory response in gingival tissues, metalloproteinase activities of neutrophils and related cytokine cascade are suppressed in the oral cavity throughout gestation (Gürsoy et al. 2010, 2014). On the other hand, in pregnancy, myeloperoxidase (MPO) accumulates at the surface of neutrophils contributing to their metabolic dynamics and enhanced release of reactive oxygen metabolites (Kindzelskii et al. 2006). In addition, according to Ssemaganda et al. (2014), part of the neutrophil populations show an activated phenotype and express arginase, which is an enzyme contributing to the T cell suppression of the mother (Kropf et al. 2007). Indeed, maternal immune responses are tightly controlled throughout pregnancy (Ssemaganda et al. 2014).

Saliva is an essential part of the defense mechanisms against continuous microbial challenge in the oral cavity. Moreover, it acts as a unique diagnostic fluid to detect infection-induced inflammatory changes in the periodontium (Sorsa et al. 2016). Numerous studies have demonstrated that, regardless of the increased gingival bleeding during pregnancy, neutrophilic chemoattractant interleukin (IL)-8, as well as IL-1 β , MMP -2, -8, -9, MPO, and elastase remain at low concentrations in saliva (Figuero et al. 2010, Gürsoy et al. 2010, 2014). Salivary levels of MMP-8 and MPO, which are strong markers of neutrophilic activity, increase after delivery (Gürsoy et al. 2010, 2014).

Salivary concentrations of antimicrobial defensins change with the progression and remission of periodontal inflammation (Guentsch et al. 2012, Pereira et al. 2013). Although cytokines and enzymes in saliva have been studied during gestation, there is no information on antimicrobial defensins and the periodontal status among pregnant women. In this study,

our hypothesis was that oral antimicrobial peptides, especially epithelial hBDs, are related to the maternal inflammatory status of periodontal tissues. Therefore, we analyzed the salivary levels of hBD-1, -2, -3, and HNP-1 in each trimester and post-partum.

Material and Methods

Pregnant and non-pregnant study populations

All participants were informed about the objectives of this cohort study, and their written consent was obtained. In order to reduce the selection bias, eligible pregnant and non-pregnant women were recruited from the Municipal Health Care Centre in Kerava, Finland. The selection criteria (Table 1) and study protocol have been previously described in detail (Gürsoy et al. 2008). Briefly, the pregnant (Pr) group (n=30) consisted of medically healthy, non-smoking Caucasian women (mean age 29.3 years, SD=2.8, range 24-35), who delivered at term (gestational age median 41 weeks, range 34-42). They were followed prospectively from the enrolment until the end of breastfeeding between October 2002 and October 2006 (Fig. 1), including three pregnancy visits during the first (Pr-T₁), second (Pr-T₂), and third (Pr-T₃) trimester, and two post-partum visits at 4-6 wks after delivery (Pr-PP₁) and after lactation ended (Pr-PP₂; mean lactation period 38.7 weeks, SD=19.2, range 8-88). The non-pregnant (NPr) group (n=24) consisted of women (mean age 30.4 years, SD=3.1, range 25-36) who were matched to the Pr group on age and the number of teeth. They had three visits (NPr₁₋₃), once per following month, which were organized in the same phase of the menstrual cycle in order to avoid potential effects of sex steroid hormone fluctuations on periodontal health (Kornman & Loesche 1980, Becerik et al. 2010). The Helsinki University Central Hospital Obstetrics and Gynecology Ethics Committee approved the study, which complied with the Declaration of Helsinki.

Data collection

All women filled a questionnaire about their education level (basic/secondary/higher), employment status (student/working/on child-care leave/unemployed), marital status (single/divorced/co-habitant/married), allergies (yes/no), smoking habits (non-smoker/smoker/former smoker), tooth brushing frequencies (once a day/twice a day/ ≥ 3 times per day), and regular dental check-ups (yes/no).

Clinical examination and saliva sampling

Oral examinations, including full-mouth periodontal measurements, were performed by one examiner (M.G.). At each visit, visible plaque index (VPI) without any disclosing solution (Ainamo & Bay 1975) and gingival bleeding on probing (BOP) were recorded from all sites of each tooth. In addition, paraffin-stimulated saliva was collected by expectoration for 5 min, and the salivary flow rate was recorded as ml/min. The samples were placed into Nunc Cryo Tubes (Thermo Fisher Scientific, Roskilde, Denmark) and stored at -70°C until their further use in the ELISA assays. All samples were analyzed blindly.

Female sex steroids in saliva

Salivary concentrations of 17β -estradiol and progesterone were determined by commercially available ELISA kits (Salimetrics, State College, PA, USA). All measurements were performed according to the manufacturer's instructions.

Salivary defensins

Commercial sandwich-ELISA kits (PeproTech® Rocky Hill, USA) were used for salivary hBD-1 and -2 detection, while hBD-3 and HNP-1 concentrations were measured by in-house sandwich-ELISA assays (Rabbit Anti-human-BD-3, cat# 500-P241; Biotinylated rabbit anti-human-BD-3, cat# 500-P241; Human BD-3, cat# 300-52; Goat anti-human-NP-1, cat#500-P126G; Biotinylated goat anti-human-NP-1, cat# 500-P126G; Human NP-1, cat# 300-42;

PeproTech® Rocky Hill, USA) according to the manufacturer's recommendations. Avidin peroxidase (SG0912, PeproTech® Rocky Hill, USA) was used as a conjugate in the protocol. All buffers and 96-well plates used were from the ELISA Buffer kit (900-K00, PeproTech® Rocky Hill, USA).

For each well, 100 µl of standard or saliva sample was used. For hBD-1, saliva was diluted to 1:30 (Diluent from ELISA Buffer kit 900-K00, PeproTech® Rocky Hill, USA). A serial diluted standard curve, as duplicate, was set for each defensin: hBD-1 from 1000 pg/ml to 15.625 pg/ml, hBD-2 from 2000 pg/ml to 31.25 pg/ml, hBD-3 from 10000 pg/ml to 312.5 pg/ml, and hNP-1 from 1000 ng/ml to 15.625 ng/ml. Also zero standards were used in every protocol. Absorbances were read with Multiskan™ EX and analyzed with the Ascent™ Software V. 2.1 (Thermo Scientific, Waltham, Massachusetts, USA).

Statistical methods

The retrospective power calculation indicated that 26 women in each group would show a mean difference of 10% (SD=15%) on BOP index using a statistical power of 90% with a significance level of 5%. Posthoc power calculation was re-calculated for the observed difference between pregnant and non-pregnant women using power calculations for mixed models (Littell et al. 2006). Based on the calculation, the study had 79% power to detect difference in hBD1. The calculation was performed with SAS Power and Sample Size 3.1 (SAS Institute Inc., Cary, NC, USA).

The primary outcomes were salivary defensins (hBD-1, -2, -3, and HNP-1), and the secondary outcomes were female sex hormones (estradiol and progesterone) and clinical parameters (VPI and BOP). Salivary defensins and female sex hormones were presented as continuous variables, while the clinical parameters were presented as categorical variables.

The normality of the continuous variables were checked with the Shapiro-Wilk test and Q-Q plot. Based on the test results, all continuous variables are presented as medians and

interquartile ranges (IQR). All categorical variables are presented as frequencies and proportions (percentages). Undetected values of each defensin (for the Pr and NPr groups HNP-1 0% and 5.9%; hBD-1 25.5% and 5.9%; hBD-2 19.7% and 2.9%; hBD-3 14.6% and 29.4%, respectively) were substituted with zero (Whitcomb & Schisterman 2008).

The multicollinearity of the predictors was checked by fitting a linear regression model with dummy response, since the response values were not involved in assessing the collinearity diagnostics. None of the condition indices indicated that collinearity existed either among the predictors, or after adjusting for the intercept.

To study the association between hormone levels and antimicrobial peptides, a generalized linear mixed model using restricted maximum likelihood estimation was fitted for each defensin separately with heterogeneous compound symmetry as a covariance structure for the repeated measurements by using hormone variables as predictors for non-pregnant women. The analysis was repeated for pregnant women adding interactions between time and hormone levels as predictors. Repeated measures mixed model was fitted to compare pregnant and non-pregnant women controlling for VPI and BOP. Each time point of pregnant women was compared to mean level of non-pregnant women. In the NPr group, the first visit's data of drop-outs (n=2) was included in the statistical analyses.

P-values less than 0.05 were considered statistically significant. All analyses were conducted using the SAS System for Windows, version 9.4TS1M1 (SAS Institute Inc., Cary, NC, USA). All figures were drawn with R 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

The distribution of the demographic, health, and oral care characteristics was similar in both groups, i.e., there were no statistical differences between pregnant and non-pregnant women in terms of their age ($p=0.253$), education level ($p=0.053$), employment status ($p=0.480$),

marital status ($p=0.227$), allergies ($p=0.166$), smoking habits ($p=0.134$), number of teeth ($p=0.961$), tooth brushing frequencies ($p=0.393$), and regular dental check-ups ($p=0.736$).

Table 2 provides the descriptive presentation of VPI% and BOP%, and concentrations of estradiol, progesterone, hBD-1, hBD-2, hBD-3, and HNP-1 in the NPr and Pr groups. None of the tested clinical or salivary parameters showed difference between three visits of the NPr group, therefore, an average value was calculated for each subject. In the Pr group, however, the levels of tested parameters at each visit are given separately.

Changes in the salivary estradiol and progesterone levels are given for the Pr group in Fig. 2. As stated above, for each hormone concentration, a single mean is given for the NPr group. When compared with the NPr group, salivary estradiol and progesterone concentrations of the Pr group were at higher levels in all three visits during pregnancy (Pr-T₁ $p<0.001$, 0.067; Pr-T₂ $p<0.001$, 0.001; Pr-T₃ $p<0.001$, <0.001 ; respectively). After delivery (Pr-PP₁ $p=0.946$, 0.945, respectively), these concentrations decreased to similar levels as seen in the NPr group (Fig. 2).

Figure 3 presents the salivary levels of hBD-1, -2, -3, and HNP-1 of the Pr and N-Pr groups. Data are presented and compared between the study groups when their concentrations are adjusted with BOP%, VPI%, and time (visits). During the first trimester (Pr-T₁), hBD-1 concentrations in saliva were at reduced levels in comparison to the NPr group ($p=0.031$). In addition, salivary hBD-1 ($p=0.012$) and HNP-1 ($p=0.031$) concentrations during the third trimester (Pr-T₃) were at lower levels than in non-pregnant women. In the Pr group, hBD-2 concentrations decreased visit by visit during pregnancy, but increased significantly ($p=0.002$) after delivery (Pr-PP₁). No change was observed in salivary hBD-3 concentrations during or after pregnancy.

Adjusted associations between the salivary defensins and BOP% and VPI% levels, and estradiol and progesterone concentrations are given in Table 3. Estimates are calculated

when time (visits) was also included into linear mixed models. According to the results, there were associations between hBD-1 and estradiol at the first trimester (Pr-T₁) and hBD-2 and progesterone at the second trimester (Pr-T₂). In the NPr group, hBD-3 associated with BOP%, while HNP-1 associated with BOP% at the second trimester (Pr-T₂) in the Pr group.

Discussion

To the best of our knowledge, this is the first study to demonstrate pregnancy-induced changes in salivary levels of antimicrobial peptides hBD-1, hBD-2, hBD-3, and HNP-1. According to the results, levels of hBD-1, hBD-2, and HNP-1 fluctuate in saliva of pregnant women and decrease especially during the third trimester. Salivary levels of hBD-3, on the other hand, stay steady during pregnancy and post-partum.

Epithelial and neutrophil defensins form an essential part of antimicrobial defense response in the oral cavity. Nevertheless, there are other antimicrobial peptides, such as cathelicidin (LL-37), adrenomedullin, beta-2-microglobulin, and calgranulin A and B, that function against continuing microbial challenge present in the mouth (Dommisch & Jepsen 2015). As our hypothesis was that epithelial and neutrophil defensin levels in saliva relate to the exaggerated inflammatory response in pregnancy, only hBD and HNP levels were measured from salivary samples. The main strength of this study is its longitudinal design, which allowed us to follow fluctuations of antimicrobial peptide levels during and after pregnancy. The use of frozen saliva samples stored for a long period may be questioned. However, we have previously tested the impact of time on the reliability of antimicrobial peptide levels by using 13-year-old salivary samples collected from periodontitis patients and periodontitis-free subjects (unpublished data) and the defensin values proved to be in line with those presented in the literature (Forte et al. 2010, Pereira et al. 2013).

In the present study, observed decreases in hBD-1, hBD-2, and HNP-1 levels in saliva during the third trimester were not related to plaque accumulation or gingival inflammation.

On the basis of these results, it is possible to claim that changes in antimicrobial peptide levels are regulated by a unique component of pregnancy, such as upregulated sex steroid hormones. Typically, the levels vary inter-individually, but mechanisms leading to this variation are not known. Estradiol modulation of the human antimicrobial peptide secretion has been demonstrated in *in vitro* studies, however, studies are few and results are conflicting. For example, when vaginal epithelial cells were stimulated by lipopolysaccharide, secretion of hBD-1 was found to be unrelated to the presence of estradiol or progesterone, while secretion of hBD-2 was increased by estradiol and decreased by progesterone (Han et al. 2010). However, in another study, hBD-2 secretion of vaginal epithelial cells was found to be reduced by estradiol, but not progesterone (Patel et al. 2013). Proinflammatory cytokines can induce hBD secretion of epithelial cells (Gursoy & Könönen 2012). Estradiol inhibits the IL-1 β stimulated hBD mRNA expression of uterine epithelial cells (Schaefer et al. 2005). In our previous studies on the same study population, we demonstrated that salivary IL-1 β and IL-8 levels stay steady during pregnancy, while dental plaque levels decrease and salivary estradiol levels increase steadily (Gürsoy et al. 2013, 2014). Infection, proinflammatory cytokines, and sex steroid hormones independently regulate the secretion of hBDs of epithelial cells. Thus, it is not possible to explain the fluctuations in hBD levels during pregnancy with the aid of *in vitro* cell experiments, where only one or two regulator(s) is tested at a time. The decrease we observed in HNP-1 levels during pregnancy is well in line with our previous study, where we found reduced levels of other neutrophilic markers, MPO and MMP-8 (Gürsoy et al. 2010). It is thereby possible to suggest that the suppression of salivary hBD-1, hBD-2, and HNP-1 during pregnancy is likely related to changes in sex steroid hormone levels. However, exact mechanisms remain unanswered.

Escribese et al. (2011) demonstrated that HNP 1-3 release by myeloid dendritic cells from pregnant women decreases or remains stable during the third trimester of pregnancy. Moreover, in cell culture studies, 17β -estradiol treatment inhibited HNP 1-3 secretion by dendritic cells (culture-generated monocyte-derived dendritic cells and directly isolated myeloid dendritic cells) in a dose-dependent manner, while progesterone did not affect alpha-defensin release (Escribese et al. 2011). Infection and inflammation increase the systemic levels of inflammatory mediators, which may cause pregnancy complications like preterm labor (Gomez-Lopez et al. 2014). Since antimicrobial peptides act as immune regulators, their suppression during pregnancy could be a protective mechanism against overexpressed inflammatory response and adverse pregnancy outcomes (Flores-Espinosa et al. 2014).

Sex steroid hormones can regulate the expression of defensins in *in vitro* conditions (Fahey et al. 2008). Moreover, cytokines, such as IL- 1β and TNF- α , can also regulate the expression of antimicrobial peptides (Menendez & Brett Finlay 2007). It has been demonstrated that the levels of defensin activating proinflammatory cytokines in oral fluids remain suppressed in pregnancy-associated gingival inflammation (Bieri et al. 2013, Figuero et al. 2010, Gürsoy et al. 2014). These results may indicate that the association between the severity of pregnancy gingivitis and antimicrobial defensin concentrations is multifactorial; there can be a direct effect of sex steroid hormones on defensin expressions, and an indirect effect through suppressed proinflammatory cytokine levels. In this study, we did not find a strong association between sex steroid hormone levels and antimicrobial defensin concentrations. One reason could be that proinflammatory cytokine levels were not included in our analyses. Yet, it is very challenging to implement all components of defensin expressions into a data analysis model. However, developing of such a study model would significantly increase our understanding on the relationship between pregnancy and antimicrobial defensins.

In the oral cavity, defensin expressions are regulated by components of infection and inflammation. The secretion of defensins stimulated by bacteria is species-specific; commensal bacteria, such as *Fusobacterium nucleatum*, can activate the secretion of hBDs from epithelial cells (Vankeerberghen et al. 2005), while two major periodontal pathogens, *Porphyromonas gingivalis* and *Treponema denticola*, can evade from the recognition by host cells or suppress the secretion of antimicrobial defensins (Shin et al. 2010). This observation, in turn, indicates that the microbial composition of dental plaque is more important than its amount, particularly when the aim is to evaluate defensin levels. In the present study, plaque scores were highest during the first trimester probably due to fact that most women suffered from nausea and gagging upon tooth brushing attempts preventing to keep up proper oral hygiene especially during the early pregnancy, and thereby leading to higher VPI scores. Therefore, we implemented VPI% to data analyses as a confounding factor to take the fluctuations of VPI into account. Our findings can be further tested and the regulatory mechanisms of defensin expressions examined by taking the levels of defensin-inducing and non-inducing bacteria separately into consideration.

In conclusion, pregnancy has suppressive effects of salivary concentrations of hBD-1, hBD-2 and HNP-1, while that of hBD-3 remains unaffected. This suggests that despite of chemical and functional similarities between these antimicrobial peptides there is an independent regulation cascade for each defensin in the oral cavity.

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Table 1. Inclusion and exclusion criteria for the pregnant (Pr) and non-pregnant (NPr) participants.

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

Table 2. Descriptive presentation of visible plaque index (VPI) and bleeding on probing (BOP) percentages, and salivary concentrations (pg/ml) of estradiol, progesterone, human beta-defensin (hBD)-1, -2, -3, and human neutrophil peptide (HNP)-1 in the pregnant (Pr) and non-pregnant (NPr) groups. Data are presented as medians and interquartile ranges.

	Pr-T₁	Pr-T₂	Pr-T₃	Pr-PP₁	Pr-PP₂	NPr
VPI %	24.71 (14.88 - 35.00)	20.46 (10.12 - 27.98)	18.08 (9.52 - 26.44)	15.52 (8.59 - 20.66)	12.80 (9.07 - 18.56)	8.07 (3.80 – 11.51)
BOP %	19.87 (9.52 - 37.22)	32.53 (22.84 - 42.78)	28.57 (22.02 - 34.48)	14.01 (8.33 - 21.99)	7.48 (5.56 - 9.43)	5.09 (2.38 – 8.75)
Estradiol (pg/ml)	20.15 (8.85 - 30.92)	45.87 (35.11 - 72.25)	49.41 (39.55 - 82.80)	2.16 (1.46 - 2.80)	2.74 (1.68 - 3.31)	2.65 (1.60 – 3.12)
Progesterone (pg/ml)	322.40 (260.80 - 454.20)	710.90 (578.40 - 1089.00)	1803.00 (1333.00 - 2283.00)	2.50 (2.50 - 9.62)	14.65 (4.39 - 35.57)	18.32 (3.63 – 89.27)
hBD-1 (pg/ml)	585.09 (0.00 - 1330.56)	1055.80 (0.00 - 2046.01)	498.39 (0.00 - 1236.25)	1167.30 (479.22 - 1566.97)	1036.96 (306.26 - 1853.32)	1623.85 (1133.68– 2243.76)
hBD-2 (pg/ml)	187.91 (64.23 - 315.57)	145.46 (31.49 - 246.90)	77.30 (0.00 - 224.06)	258.79 (46.42 - 451.45)	169.96 (95.04 - 282.19)	170.95 (89.74 – 289.27)
hBD-3 (pg/ml)	656.36 (517.86 - 803.88)	567.95 (462.40 - 727.39)	605.58 (0.00 - 941.97)	673.28 (478.68 - 959.02)	760.04 (581.76 - 1079.29)	415.66 (0.00 – 987.95)
HNP-1 (pg/ml)	33.52 (15.35 - 80.34)	36.97 (16.63 - 79.26)	25.31 (12.19 - 65.69)	64.22 (22.95 - 131.86)	34.63 (16.84 - 77.60)	43.26 (26.70 – 129.72)

T₁, the first trimester; T₂, the second trimester; T₃, the third trimester; PP₁, 4-6 wks after delivery; PP₂, after lactation ended

Table 3. Adjusted associations between the antimicrobial peptides (hBD-1-3 and HNP-1) in saliva, clinical parameters, and hormone levels of the pregnant (Pr) and non-pregnant (NPr) groups. Estimates and confidence intervals (CI) are calculated when time (visits) was also included in generalized linear mixed models.

		NPr			Pr-T ₁			Pr-T ₂			Pr-T ₃		
		estimate	CI	<i>p</i>	estimate	CI	<i>p</i>	estimate	CI	<i>p</i>	estimate	CI	<i>p</i>
hBD-1	BOP	-55.3	-164 – 53.6	0.527	13.1	-17.9 – 44.1	0.640	-7.20	-48.6 – 34.2	0.960	-4.53	-39.1 – 30.1	0.982
	VPI	3.10	-76.3 – 82.5	0.999	-13.2	-52.9 – 26.6	0.790	-10.3	-59.3 – 26.6	0.934	6.90	-36.1 – 49.9	0.968
	Estradiol	-213	-470 – 43.1	0.127	-39.2	-76.9 – -1.52	0.039	2.65	-14.7 – 19.9	0.976	5.06	-10.3 – 20.4	0.810
	Progesterone	-1.76	-5.76 – 2.24	0.639	1.81	-0.74 – 4.37	0.237	0.18	-0.56 – 0.93	0.900	-0.09	-0.43 – 0.26	0.894
hBD-2	BOP	13.3	-10.3 – 36.9	0.420	2.54	-2.96 – 8.04	0.567	1.06	-8.01 – 10.1	0.986	-1.38	-6.03 – 3.26	0.826
	VPI	-5.27	-21.4 – 10.8	0.826	0.14	-7.02 – 7.30	0.999	-5.19	-16.1 – 5.74	0.542	0.43	-5.38 – 6.24	0.996
	Estradiol	-25.8	-73.4 – 21.8	0.455	4.27	-1.51 – 10.05	0.213	-1.26	-4.97 – 2.45	0.795	0.60	-1.42 – 2.61	0.852
	Progesterone	-0.10	-0.91 – 0.71	0.994	-0.05	-0.40 – 0.30	0.974	0.22	0.07 – 0.38	0.002	-0.01	-0.05 – 0.04	0.961
hBD-3	BOP	88.6	10.9 – 166	0.020	6.25	-5.19 – 17.7	0.433	-3.55	-16.2 – 9.13	0.848	-10.2	-30.2 – 9.76	0.487
	VPI	-23.6	-77.1 – 29.9	0.641	-1.69	-16.5 – 13.2	0.986	3.65	-11.6 – 18.9	0.898	1.25	-23.7 – 26.17	0.999
	Estradiol	-46.7	-206 – 112	0.871	8.38	-3.62 – 20.4	0.253	3.89	-1.29 – 9.07	0.198	5.07	-3.57 – 13.72	0.390
	Progesterone	-1.56	-4.26 – 1.13	0.402	-0.42	-1.15 – 0.30	0.384	-0.05	-0.27 – 0.18	0.938	-0.16	-0.36 – 0.04	0.142
HNP-1	BOP	-4.41	-26.4 – 17.6	0.960	1.11	-2.10 – 4.32	0.750	3.58	1.14 – 6.02	0.002	0.45	-1.52 – 2.41	0.912
	VPI	8.95	-6.46 – 24.4	0.404	0.45	-3.66 – 4.56	0.988	-0.19	-3.07 – 2.69	0.997	0.17	-2.27 – 2.62	0.997
	Estradiol	0.57	-46.1 – 47.3	1.000	3.74	-0.20 – 7.68	0.067	0.78	-0.25 – 1.81	0.182	0.12	-0.75 – 0.99	0.981
	Progesterone	0.25	-0.52 – 1.03	0.830	-0.15	-0.42 – 0.11	0.387	-0.02	-0.06 – 0.03	0.750	0.00	-0.02 – 0.02	0.969

T₁, the first trimester; T₂, the second trimester; T₃, the third trimester. One unit increase in clinical parameters or hormone levels is associated with

“estimate X unit” decrease (negative value) or increase (positive value) in tested antimicrobial peptide. For example in N-Pr group, one unit increase in BOP is associated with 55 unit decrease in hBD-1.

Figure legends

Fig. 1. Study design and flow.

Fig. 2. Changes in salivary estradiol and progesterone levels during pregnancy (Pr-T₁₋₃) and post-partum (Pr-PP₁₋₂). In the non-pregnant (NPr) group, an average value of three visits was calculated for each subject and presented. *** $p < 0.001$

Fig. 3. The salivary levels of human beta-defensin (hBD)-1, -2, -3, and human neutrophilic peptide (HNP)-1 of the pregnant (Pr) and non-pregnant (N-Pr) groups are given as adjusted medians (grey areas indicate 95% Confidence Intervals). Data is presented and compared between the groups when their concentrations are adjusted with visible plaque index (VPI%), bleeding on probing (BOP%), and time (visits).