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# Longitudinal study of salivary proteinases during pregnancy and postpartum

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*Background and Objective:* Matrix metalloproteinases (MMPs) and their regulators are connected to periodontal inflammation and destruction. However, the presence and role of the salivary MMPs in pregnancy-related gingivitis are not well known. Our longitudinal study aimed to monitor salivary proteinase levels and possible changes, and relate them to periodontal status during pregnancy and postpartum.

*Material and Methods:* Salivary samples were collected from 30 periodontally healthy pregnant women five times (once during each trimester, 4–6 wk after delivery and after lactation) and, as their controls, from 24 non-pregnant women three times (during successive months). Periodontal examination included visible plaque index, bleeding on probing, probing pocket depth and clinical attachment level measurements. Matrix metalloproteinase-8 levels were measured by immunofluorometric assay, and MMP-2 and MMP-9 levels and molecular forms by gelatin zymography. Salivary elastase, myeloperoxidase and tissue inhibitor of matrix metalloproteinase-1 levels were measured by ELISA.

*Results:* Elastase concentrations maintained stable during the follow-up, while myeloperoxidase concentrations increased significantly after delivery. During pregnancy, MMP-8 concentrations were significantly lower than postpartum concentrations, being lowest during the second trimester and highest after delivery, and varying inversely to pregnancy gingivitis, observed as elevated percentages of bleeding on probing and probing pocket depth during the second and third trimester. In pregnant women, the highest MMP-2 and MMP-9 levels were found in saliva after lactation. In the control group, both clinical and enzymological findings remained stable during the follow-up period.

*Conclusion:* Our results suggest that hormonal changes during pregnancy induce or enhance susceptibility to gingivitis, while salivary proteinase and myeloperoxidase levels are reduced.

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Pregnancy increases the propensity to gingival inflammation, which is known as pregnancy gingivitis (1–3). Besides an enhanced gingival bleeding tendency without specific plaque association, periodontal pocket formation can increase during pregnancy (3,4). Those pregnancy-related periodontal changes, however, seem to be reversible after delivery. Therefore, pregnancy gingivitis may not predispose or progress to periodontitis, as classified by increased pocket depth and loss of clinical attachment. This has been demonstrated recently in two longitudinal studies with different postpartum follow-up periods (2,3).

The exact etiology of pregnancy gingivitis is still unknown, although elevated concentrations of female sex hormones during pregnancy are

thought to have a major role in the occurrence of gingivitis (5). In addition to the direct physiological effects on the periodontium, the increased concentrations of female sex hormones in plasma during pregnancy may modulate the function and activity of neutrophils/polymorphonuclear (PMN) leucocytes (6–8). Therefore, since PMN cells exert an important role in the inflammatory response, elevated hormonal concentrations may change this defense mechanism of the body during pregnancy, which is seen as increased susceptibility to infection. Furthermore, Lapp *et al*. (9) demonstrated recently in their *in vitro* study that progesterone is able to reduce the matrix metalloproteinase production by cultured human gingival fibroblasts in response to interleukin-1β.

Matrix metalloproteinases (MMPs) are a group of genetically distinct but structurally related proteolytic enzymes, which are able to degrade almost all extracellular matrix components in normal and pathological tissue remodeling (10,11). They have many important roles during different gestational stages, being involved with trophoblast invasion (12), embryo implantation and decidual development (13) in early gestation, and fetal membrane rupture (14) in parturition. In contrast, MMPs and their regulators are usually connected to periodontal inflammation and destruction (15,16). However, their presence in saliva during pregnancy and their role in pregnancy-related gingivitis are little known. Our hypothesis was that hormonal changes during pregnancy have an impact on periodontal tissues by enhancing growth of certain anaerobic bacteria and reducing neutrophilic activities. According to our previous study (3), reporting the clinical changes in the same study groups, pregnant women had a high tendency of gingival inflammation during the second trimester, regardless of the amount of plaque. Hence, an increased neutrophilic activity in saliva is expected during the second trimester of pregnancy. Therefore, as part of our investigation of pregnancy-related events in the oral cavity, the present longitudinal study aimed to monitor salivary proteinase levels and their possible changes, and compare them to periodontal status at different phases of pregnancy and postpartum.

## Material and methods

### Study and control populations

Thirty generally and periodontally healthy, non-smoking, Caucasian pregnant women (the Pr group) and 24 non-pregnant women as their controls (the N-Pr group) were recruited into the study. A brief summary of various characteristics of these two populations is provided in Table 1. A more detailed description of these two groups has been given by Gürsoy *et al*. (3). Briefly, the women in the Pr group had check-ups three times during pregnancy (Pr Ex I at 12–14 wk, Pr Ex II at 25–27 wk and Pr Ex III at 34–38 wk of pregnancy) and twice postpartum (Pr Ex IV at 4–6 wk after delivery and Pr Ex V after lactation, when breastfeeding had ended). The women in the N-Pr group were seen three times (N-Pr Ex I, N-Pr Ex II and N-Pr Ex III), once in each successive month. The women in both groups did not receive any periodontal treatment procedures during the follow-up visits. The Helsinki University Central Hospital Obstetrics and Gynecology Ethics Committee reviewed and approved the research protocol. The study complied with the Declaration of Helsinki. All participants were informed of the purpose and objectives of the study, and written informed consent was obtained from each subject.

*Table 1*. Summary of the characteristics of the women in the pregnant (Pr) and non-pregnant (N-Pr) groups

| | The Pr group | The N-Pr group |
|---|---|---|
| | Mean ± SD (range) | Mean ± SD (range) |
| Age (years) | 29.3 ± 2.8 (24–35) | 30.4 ± 3.1 (25–36) |
| Number of teeth | 28.5 ± 1.4 (25–32) | 28.6 ± 1.8 (24–32) |
| Duration of pregnancy (wk) | 40.1 ± 2.1 (34–42) | — |
| Duration of lactation (wk) | 38.7 ± 19.2 (8–88) | — |

### Clinical measurements and sample collection

Periodontal status, based on visible plaque index (VPI), bleeding on probing (BOP), probing pocket depth and clinical attachment level (CAL) measurements, was recorded during each visit as described previously (3). All clinical measurements were examined from six sites per tooth, including third molars. Paraffin-stimulated saliva was collected by expectoration for 5 min, and the sample was divided and placed into two plastic Eppendorf tubes. The samples were transported to the Institute of Dentistry, Biomedicum, Helsinki, within 2 h and stored immediately at −20°C until assayed.

### Immunofluorometric assay

Salivary MMP-8 levels were detected by time-resolved immunofluorometric assay with specific monoclonal antibodies (17). The monoclonal MMP-8 specific antibodies 8708 and 8706 (Medix Biochemica, Kauniainen, Finland) were used as a catching antibody and a tracer antibody, respectively. The tracer antibody was labeled using europium-chelate (18). The assay buffer contained 20 mM Tris–HCl (pH 7.5), 0.5 M NaCl, 5 mM $CaCl_2$, 50 μM $ZnCl_2$, 0.5% bovine serum albumin, 0.05% sodium azide and 20 mg/L diethylenetriaminepentaacetic acid. Salivary samples were diluted in assay buffer and incubated for 1 h, followed by incubation for 1 h with tracer antibody. Enhancement solution was added and, after 5 min, fluorescence was measured using a 1234 Delfia Research Fluorometer (Wallac, Turku, Finland). The specificity of the monoclonal antibodies against MMP-8 corresponded to that of polyclonal

MMP-8 (19). Data are expressed in ng/mL.

### Gelatinolytic zymography

The presence and activities of two gelatinases, 72 kDa MMP-2 (gelatinase A) and 92 kDa MMP-9 (gelatinase B), were measured by gelatin zymography (15,20). Salivary samples were incubated with the sample buffer for 2 h at room temperature in the dark, and then loaded to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) gels containing 1 mg/mL of fluorescent 2-methoxy-2,4-diphenyl-3-(2*H*)-furanone (MDPF) gelatin (Fluka, Buchs SG, Switzerland) as substrate (20). Electrophoresis was carried out for 1.5 h at 4°C with constant voltage of 110 V. After electrophoresis, the gels were first washed and incubated for 30 min in 50 mM Tris–HCl (pH 7.5), including 0.02% (w/v) $NaN_3$ and 2.5% Tween 80, and then for 30 min in the same buffer, supplemented with 5 μM $ZnCl_2$ and 1 mM $CaCl_2$. Finally, the gels were incubated overnight for 18 h at 37°C in 50 mM Tris–HCl (pH 7.5), including 0.02% (w/v) $NaN_3$, 5 μM $ZnCl_2$, and 1 mM $CaCl_2$. After incubation, the degradation of gelatin was visualized under long-wave ultraviolet light, and then the gels were fixed and stained with 0.1% Coomassie Blue R-250 in 30% methanol and 10% acetic acid. The gelatinolytic levels and activities were visualized as clear bands against the blue background. The levels of MMP-2 and MMP-9 were determined using a scanning densitometer with Kodak molecular imaging software (Rochester, NY, USA) and expressed as relative levels derived from densitometric units (20,21). Molecular weights of the gelatinolytic activities were confirmed using prestained low-range SDS–PAGE standards (Bio-Rad, Hercules, CA, USA).

### Enzyme-linked immunosorbent assay analyses

Elastase, myeloperoxidase (MPO) and TIMP-1 levels in saliva were assessed by using commercial ELISA kits: Human PMN elastase (Bender MedSystems Inc., Burlingame, CA, USA), MPO ELISA Kit (Immunodiagnostik AG, Bensheim, Germany) and Amersham TIMP-1, Human, Biotrak, ELISA system (GE Healthcare, Amersham, Buckinghamshire, UK), respectively. All salivary samples were diluted with sample dilution buffer for elastase (1:100), for MPO (1:20) and for TIMP-1 (1:10). The following procedure steps were performed according to the manufacturers' instructions. The optical densities were determined at 450 nm using the Labsystems Multiskan MS microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). Data are expressed in ng/mL.

### Statistics

All data are shown as medians with interquartile range. Statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). The differences between the follow-up visits within the two populations were identified first with the Friedman test and then compared using the Wilcoxon signed ranks test. Furthermore, for the comparison of the values between the two groups, the Mann–Whitney *U* test was used. The level at which comparisons were accepted as statistically significant was set at $p$-values $< 0.05$.

## Results

A total of 205 saliva samples were collected and analyzed. A few missed visits during the follow-up were the reason for some unavailable samplings. The number of subjects per visit is presented in Table 2. In the N-Pr group, both clinical and all enzymological findings remained relatively stable during the follow-up period. Therefore, these results are shown as one median value (N-Pr Ex) calculated from three successive visits (N-Pr Ex I–III).

During pregnancy, salivary MMP-8 concentrations were significantly lower than postpartum concentrations, being lowest during the second trimester and highest after delivery (Fig. 1A), i.e.

*Table 2.* Comparison of the clinical parameters assessed from six sites per tooth in the pregnant (Pr) study population at five visits (Pr Ex I–Pr Ex V) and in the non-pregnant (N-Pr) control population

| Visit | No. of subjects | VPI (% median) (IQ) | BOP (% median) (IQ) | No. of pockets (≥ 4 mm) (median) (IQ) | No. of teeth with loss of CAL (median) (IQ) |
|---|---|---|---|---|---|
| Pr Ex I | 29 | 24.71 (14.44–35.95) | 19.87 (9.52–37.29) | 0.00 (0.00–0.00) | 0.00 (0.00–0.00) |
| Pr Ex II[a] | 30 | 20.46 (9.97–28.20)** | 32.53 (22.49–42.80)** | 9.50 (3.75–17.25)*** | 0.00 (0.00–0.00) |
| Pr Ex III[b] | 26 | 18.08 (9.23–26.50)* | 28.57 (20.69–34.61)** | 5.50 (0.75–12.00) | 0.00 (0.00–0.00) |
| Pr Ex IV[c] | 28 | 15.52 (8.17–21.05) | 14.01 (8.33–23.79)*** | 0.00 (0.00–3.00)** | 0.00 (0.00–0.00) |
| Pr Ex V[d] | 24 | 12.80 (8.85–18.61) | 7.48 (5.56–9.48)** | 0.00 (0.00–0.00)* | 0.00 (0.00–0.00) |
| N-Pr Ex[e] | 24 | 8.47 (3.92–12.10)** | 5.75 (2.83–7.76) | 0.00 (0.00–0.00) | 0.00 (0.00–0.00) |

Abbreviations: IQ, interquartile range, 25th–75th percentile; VPI, visible plaque index; BOP, bleeding on probing; and CAL, clinical attachment level.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

[a] Significant difference between Pr Ex I and Pr Ex II.

[b] Significant difference between Pr Ex II and Pr Ex III.

[c] Significant difference between Pr Ex III and Pr Ex IV.

[d] Significant difference between Pr Ex IV and Pr Ex V.

[e] Significant difference between Pr Ex V and N-Pr Ex.

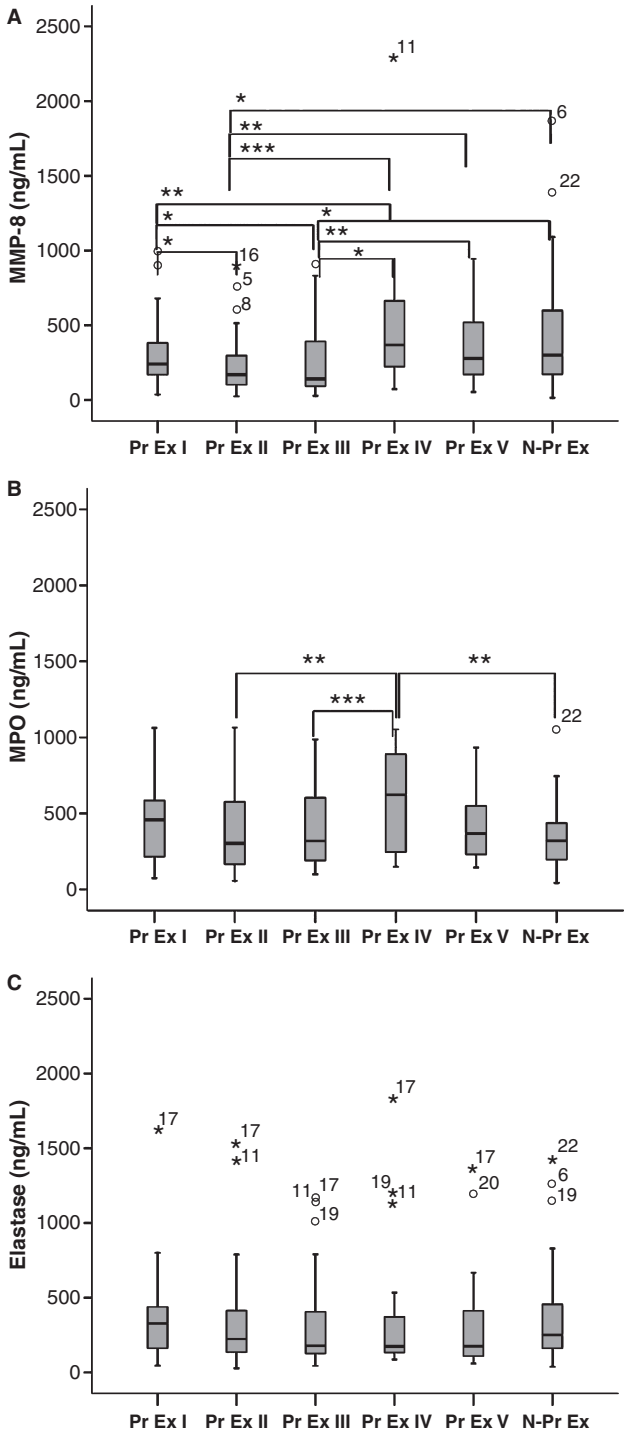


*Fig. 1.* Comparison of MMP-8 (A), MPO (B) and elastase levels (C) in saliva from pregnant (Pr) women at five visits (Pr Ex I–Pr Ex V) and non-pregnant (N-Pr) women (N-Pr Ex). The MMP-8 values were measured with time-resolved immunofluorometric assay, and MPO and elastase with ELISAs. The box plots indicate the median (horizontal line), quartiles (boxes) and non-outlier values (whiskers). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

varying inversely to pregnancy gingivitis seen as elevated percentages in BOP and probing pocket depth during the second and third trimester (Table 2). After delivery, there were no statistically significant differences in MMP-8 concentrations between the Pr and N-Pr groups (Fig.1A). Myeloperoxidase concentrations were also significantly lower during the second and third trimesters, increasing significantly ($p < 0.0001$) after delivery (Fig.1B). Elastase concentrations maintained stable during the follow-up, and they did not differ between the Pr and N-Pr groups (Fig.1C).

During pregnancy and after delivery, salivary TIMP-1 concentrations maintained stable. However, the levels were significantly lower than those in the N-Pr group (Fig. 2A). After lactation, TIMP-1 concentrations increased to the same level as observed in the N-Pr group. The MMP-8/TIMP-1 molar ratios were significantly lower during pregnancy, decreasing visit by visit throughout pregnancy and being at the lowest level during the third trimester (Fig. 2B). The highest ratio was reached after delivery; after lactation this returned to the same level as observed in the N-Pr group.

In the Pr group, MMP-9 levels were significantly ($p < 0.05$) lower, while MMP-2 levels did not differ in comparison to those of the N-Pr group. In the Pr group, the highest salivary MMP-2 and MMP-9 levels were found after lactation (Fig. 3).

## Discussion

Pregnancy increases the susceptibility to gingival inflammation (3). Although proteinases and their regulators have major roles in periodontal diseases, and also pregnancy-related hormones are found to diminish proteinase responses (6–9), there are hardly any published data on changes in salivary proteinases during pregnancy. Previous studies, defining MMP and TIMP expressions during pregnancy and/or parturition, have used gestational tissues, serum, plasma and/or urine as study specimens (14,22–25). In a recent cross-sectional study, Menon *et al.* (26) aimed to find patients at risk for preterm premature rupture of the membranes by detecting salivary proteinase activities. However, their interpretation is incomparable, since no assessments were made of clinical periodontal or oral status of standard patient and control groups. Therefore, according to our knowledge, the

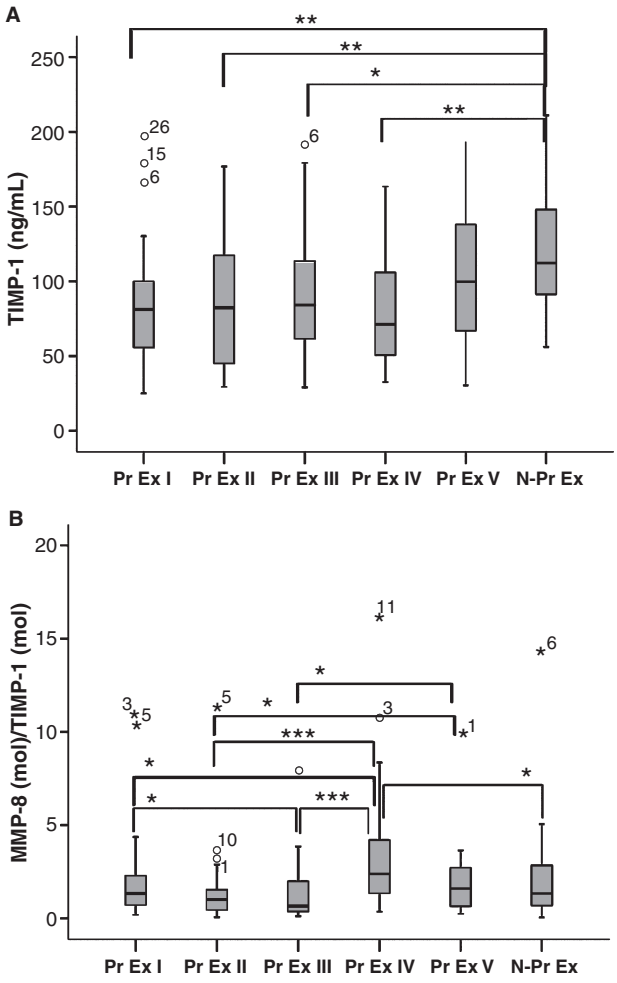


*Fig. 2.* Comparison of TIMP-1 levels (A) and MMP-8/TIMP-1 molar ratios (B) in saliva from Pr women at five visits (Pr Ex I–Pr Ex V) and N-Pr women (N-Pr Ex). The box plots indicate the median (horizontal line), quartiles (boxes), and non-outlier values (whiskers). $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

present study is the first longitudinal study analyzing salivary proteinases and their regulators during pregnancy and postpartum, and comparing them with clinical periodontal status. As a result, our present work provides new data on the occurrence of these enzymes in saliva and their relationship with the periodontal changes during pregnancy.

Our results indicate that pregnancy-associated hormonal changes reflect the reduced expression and activity of several proteinases not only in periodontal tissues but also in saliva. A significant reduction of salivary MMPs, MPO and TIMP-1 expression occurred during pregnancy, and then levels returned to normal after lactation. Since neutrophils are involved in proteolysis and thereby contribute to the development of tissue destruction in periodontitis, the absence of initiation of periodontitis throughout gestation could, at least in part, be explained by the inhibitory effects of pregnancy on neutrophil functions. It is conceivable that low levels of the tested enzymes may be due to their decreased release from neutrophils. This can be considered for MPO, MMP-8 and elastase, because immunodetection techniques were used in the present study. However, the detection of MMP-2 and MMP-9 by gelatin zymography does not allow us to analyze extracellular and intracellular enzymes separately. Hence, this hypothesis cannot be proven within the limits of the present study.

Pregnancy is known to affect neutrophils by decreasing their bactericidal activities, such as chemotaxis (6,27) and phagocytosis (28,29). Reduced microbial killing by neutrophils has been observed at the end of the first trimester (7), and the other suppressive effects on neutrophil functions, mentioned above, at the beginning of the second trimester (27,29), persisting throughout pregnancy. In pregnant women, neutrophil responses seem to return to the same level as seen in non-pregnant control subjects by 6 wk after delivery (7,8); however, a longer recovering time, up to 3 mo after parturition, has also been detected (27).

Matrix metalloproteinase-8, MMP-9, elastase and MPO are mainly derived from and released by neutrophils (30) and thereby neutrophils are the main source of these enzymes in the oral cavity and in whole saliva (15,31–34). Impairment of neutrophil functions during pregnancy may partly explain why salivary MMP-8 and MPO concentrations, in particular, but also that of MMP-9, were found to be reduced in the Pr group. In contrast, there are various sources, such as bacteria, macrophages and epithelial cells, which are able to produce proteinases in the oral cavity (16). For example, the origin of MMP-2 in periodontal tissues is mainly from gingival fibroblasts (10,11,35). Thus, it can be speculated that the origin of the proteinases we have measured in saliva may be from cell types other than neutrophils. It should also be kept in mind that, in the present study, saliva was collected by paraffin stimulation. Hence, collected saliva contained a higher proportion of glandular saliva, affecting gingiva-derived components. However, the longitudinal curves of salivary proteolytic enzyme concentrations corresponded to neutrophilic function impairments illustrated in earlier studies, as we described above. Hence, we deduce that reduced salivary proteolytic levels can be the outcome of impaired neutrophilic functions during pregnancy. Furthermore, according to

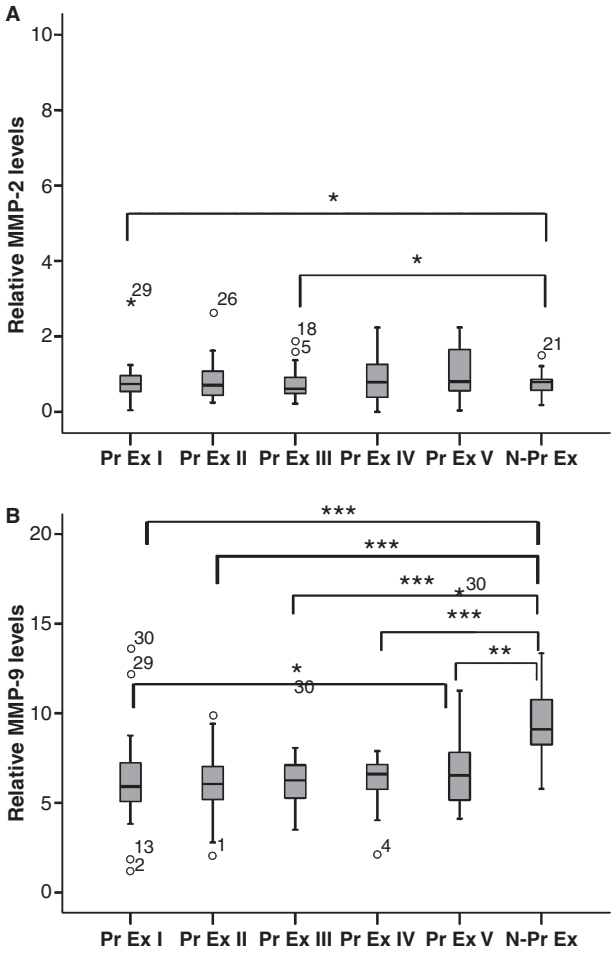


*Fig. 3.* Comparison of MMP-2 (A) and MMP-9 levels (B) in saliva from Pr women at five visits (Pr Ex I–Pr Ex V) and N-Pr women (N-Pr Ex). Values are expressed as relative levels derived from densitometric units. The box plots indicate the median (horizontal line), quartiles (boxes) and non-outlier values (whiskers). $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Miyagi *et al.* (36), female sex hormones play a role in altered neutrophil chemotaxis, without any effect on the chemotaxis of monocytes.

In the present study, salivary MPO concentrations decreased after the first trimester, stayed at low levels throughout pregnancy, increased to their highest levels after delivery and, finally, returned to normal levels after lactation. Similar changes have been observed in venous blood MPO levels; in a study on peripheral blood neutrophil function at various intervals throughout pregnancy and up to 5–6 wk postpartum, MPO levels in venous blood stayed low during pregnancy but increased after delivery (7). Owing to the similarities between the salivary and venous MPO levels during pregnancy, it can be anticipated that the effect of hormones on MPO are more likely to be systemic than local. Degranulation of the polymorphonuclear leukocytes within the circulation as a response to circulating immune complexes was proposed as one possible mechanism responsible for the MPO reduction (7). Öberg *et al.* (37), however, demonstrated that the cellular MPO content in polymorphonuclear leukocytes remains unchanged, while peroxidase activity is reduced during pregnancy. Unfortunately, their methods were not able to separate neutrophils from eosinophils. They explained the reduction in MPO activity by the decrease in eosinophil peroxidase, which was an outcome of the decreased number of eosinophils during pregnancy.

In the Pr group, the elastase levels were relatively stable throughout the follow-up period and did not differ from those of the non-pregnant control subjects. The reason for this remained unclear. In the present study, ELISA was used to detect salivary elastase. Since ELISA measures the elastase–$\alpha_1$-proteinase inhibitor complex but not the free enzyme or its complex with $\alpha_2$-macroglobulin, this may partly explain the unaffected levels of salivary elastase, in contrast to other neutrophilic enzymes. Neutrophil elastase is usually found in saliva of periodontitis patients, the enzyme activity being associated with disease status and severity (33,34). Thus, the low elastase concentrations support our clinical findings, i.e. all subjects proved to be periodontally healthy in the end of the follow-up (3). In fact, other proteinase concentrations also returned after pregnancy to the level seen in non-pregnant women, indicating that, in periodontally healthy women, pregnancy itself does not predispose to periodontitis. Furthermore, we compared our salivary MMP-8 data with those available from a subgroup of 165 adults of the Finnish national health examination survey 'Health 2000' (U. K. Gursoy, E. Könönen, P. Pradhan-Palikhe, T. Tervahartiala, P. J. Pussinen, L. Suominen-Taipale, M. Knuuttila, T. Sorsa, unpublished data); the present MMP-8 levels in the pregnant and control groups were significantly lower than those in periodontitis subjects, but similar to those in subjects without periodontal pockets. Interestingly, physiological MMP-8 levels in periodontal tissues have recently been demonstrated to be protective or anti-inflammatory (38).

According to Ingman *et al.* (16), in stimulated saliva, TIMP-1 originates mainly from the salivary glands rather than gingiva or gingival crevicular fluid. In our study, TIMP-1 levels, but not the MMP-8/TIMP-1 ratio, were significantly lower in pregnant women than in the control subjects. Pregnancy-associated hormonal changes down-regulate salivary TIMP-1 levels.

A similar observation was made in a cross-sectional study by Clark *et al*. (22), who analyzed serum TIMP-1 levels in women with full-term pregnancies (37–42 wk). They reported significantly lower TIMP-1 levels in serum throughout gestation up to 37 wk of pregnancy in comparison to those in non-pregnant control subjects.

In conclusion, hormonal changes seem to induce or enhance susceptibility to gingivitis during pregnancy, when salivary proteinase and MPO levels are decreased. Reduced proteolysis in periodontal tissues throughout gestation and its return to normal levels after delivery may explain, at least in part, why the periodontal inflammation during pregnancy is limited to gingivitis and does not predispose or proceed to periodontitis. Our further studies, using the same study population, aim to analyze proteolytic enzyme activities in gingival crevicular fluid and to compare them with site-specific measurements of pocket depth.

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