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Differential elevation of matrix metalloproteinase expression in women exposed to levonorgestrel-releasing intrauterine system for a short or prolonged period of time

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Abstract:
BACKGROUND: The levonorgestrel-releasing intrauterine system (LNG-IUS) is an effective contraceptive and has many non-contraceptive health benefits. However, it is commonly associated with irregular endometrial bleeding. Metalloproteinases contribute to extracellular matrix (ECM) remodelling and regulate bleeding during the menstrual cycle. Enhanced metalloproteinase expression participates in the pathogenesis of breakthrough bleeding. Thus the objective of this study was to compare matrix metalloproteinase (MMP) expression in endometrium during luteal phase and in short-term (I month) and long-term (≥ 6 months) LNG-IUS users.
METHODS: MMP expression was analysed by semi-quantitative RT-PCR and immunohistochemistry. Gelatinase activity was determined by gelatin zymography.
RESULTS: MMP-1, -2, -3, -7, -9 and -12 mRNAs levels were increased, whereas that of MMP-26 was decreased in the endometrium of LNG-IUS users. MMP-1, -2, -3, -7 and -9 were localized by immunohistochemistry in all biopsies in the short-term group but in only 0-27% in the control group. The incidence of positive immunostaining for MMP-2 and -3 decreased significantly in the long-term compared with short-term LNG-IUS users. MMP-26 was localized in all biopsies from the control group but in only 14 and 25% from the short- and long-term LNG-IUS groups, respectively. In both LNG groups, the numbers of macrophages (the major source of MMP-12) was increased.
CONCLUSIONS: MMP-1, active MMP-2, MMP-3, MMP-7, MMP-9 and MMP-12 are more prevalent in the short-term LNG-IUS group, suggesting their important contribution to ECM breakdown and transient bleeding. The decrease in the percentage of women expressing MMP-2 and -3 might contribute to the decreased occurrence of unwanted spotting and bleeding in long-term LNG-IUS users.

Key words: levonorgestrel-IUS ; matrix metalloproteinases ; endometrial bleeding ; contraceptive

INTRODUCTION

The progestogen-only contraception methods are used by a considerable number of women. The 20 µg/day levonorgestrel- releasing intrauterine system (LNG-IUS) is associated with irregular and abnormal endometrial bleeding which constitutes one of the most common reasons for discontinuation of their use. Spotting and breakthrough bleeding are more frequently documented during the first few months of treatment. A multitude of factors have been proposed to contribute to this disorder but the exact mechanism leading to the bleeding episodes remains to be elucidated.

Among these factors, the tissue factor, the granulocyte-macrophage colony-stimulating factor and angiogenic factors have been reported (Critchley et *al.*, 1998; Zhu et *al.,* 1999; Lockwood et *al*., 2000; Laoag-Fernandez et *al.,* 2003; Roopa et *al.,* 2003; Mints et al., 2005). In addition, progesterone receptor and estrogen receptor expressions are dramatically reduced in endometrial epithelial and stromal cells of LNG-IUS users (Zhu et al., 1999; Jones and Critchley, 2000; Galant et *al.,* 2004; Vereide et *al.,* 2006). An important modification in endometrial microvascular density and decrease in vessel maturation due to a lack of surrounding pericytes/smooth muscle cells is probably involved in this bleeding process along with modifications of endothelial basement membrane structure and composition (Rogers et *al.,* 1993; Hickey et *al.,* 1999; Jondet et *al.,* 2005; Stephanie et al., 2007).

The matrix metalloproteinases (MMPs) represent one of the main candidates that play a critical role in these bleeding disorders (Skinner et *al.,* 1999; Vincent et *al.,* 1999; Galant et *al.,* 2000; Chegini et *al.,* 2003; Galant et *al.,* 2004). In endometrium, MMPs are involved in matrix remodelling associated with the perimenstrual phase (Kokorine et *al.,* 1996; Marbaix et *al.,* 1996; Salamonsen et *al.,* 2002). Synthesized as inactive zymogens, they are either secreted or expressed as transmembrane proteins, they are inhibited by tissue inhibitors of MMPs and they can hydrolyse extracellular matrix (ECM) and non-ECM proteins (such as adhesion molecules, growth factors and cytokines). The MMP family is divided into four enzyme groups: collagenases, stromelysins, gelatinases and membrane-bound types, with a broad range of ECM substrates (Woessner, 1994; Nagase and Woessner, 1999). MMPs are differentially expressed during the various phases of the menstrual cycle. The expression of MMP-1 (collagenase-1), MMP-2 (gelatinase A), MMP-3 (stromelysin-1), MMP-7 (matrilysin-1), MMP-9 (gelatinase B) and MMP-12 (metalloelastase) is increased during menstruation in contrast to MMP-26 (matrilysin-2), which is expressed more during the proliferative phase (Goffin et al., 2003). Previous results have shown that MMP-9 is up-regulated in stromal and epithelial cells following the administration of LNG-IUS (Skinner et al., 1999). Inflammatory cells could also play a role in irregular bleeding in LNG-IUS and Norplant users by secreting an increased level of MMP-1 and MMP-9 (Vincent *et al.,* 1999; Milne et *al.,* 2001).

This study was aimed at assessing the expression of MMPs (specifically MMP-1, -2, -3, -7, -9, -12 and -26), most of which are up-regulated during normal menstruation, in endometrial biopsies from women with an LNG-IUS for 1 month or for 6 months to 5 years.

MATERIALS AND METHODS

Patients

The study was approved by the Ethical Committee of Liège University Hospital. There were 37 endometrial biopsies analysed. Patients treated with LNG-IUS for 1 month or for 6 months or more are referred to as 'short-term' and 'long-term' treated patients, respectively. Of the biopsies, 14 were obtained from women exposed to LNG-IUS (Mirena , Bayer Schering Pharma, Berlin, Germany) for a period of 1 month. For this purpose, seven women (mean age 40.5 ± 4.6 years) with an indication of hysterectomy for repetitive episodes of menorrhagia were prospectively recruited. Written informed consent was obtained. These patients underwent an office hysteroscopy between days 21 and 25 of a spontaneous cycle without a bleeding episode, in order to establish the aetiology of the intermittent menorrhagia and to exclude malignancy. In all cases, the uterine cavity was found to be covered by endometrium without polyps myoma or malignancy. An endometrial biopsy was simultaneously performed with a Cornier Pipelle suction curette (baseline biopsy), and ovulation was documented by plasma determination of progesterone above 6 ng/ml. A second biopsy was taken at the time of hysterectomy. LNG-IUS was inserted for a median period of I month prior to surgery during which all women experienced one or several spotting/bleeding episodes for a total of 15-21 days duration (short-term LNG group).

Twelve biopsies were obtained from women (mean age 40.1 ± 6.7 years) exposed to LNG-IUS for a long period [mean duration of exposure 33 months (range 6-60 months)]. In three women, an office hysteroscopy was performed for transient bleeding that had occurred more than 40 days before endometrial sampling. The endometrium was found to be atrophic with no intrauterine pathology. In nine other cases, endometrial biopsies were performed at the time of LNG-IUS withdrawal for fertility desire or LNG-IUS change (long-term LNG group). In both LNG groups, the absence of intrauterine organic lesions was thus documented.

The control group consisted of I I endometrial biopsies, performed during the mid-secretory phase of a spontaneous documented ovulatory cycle of healthy women with documented fertility (mean age 33.1 + 3.1 years). This control group allowed an adequate comparison between LNG-IUS users and control women with 'normal endometrium', spontaneous ovulatory cycles and documented fertility. This group is more appropriate than an age-matched control group. It indeed allows the comparison of the impact of LNG on endometrial histology with that of spontaneous ovulatory cycles during the luteal phase with optimal exposure to endogenous progesterone. On the contrary, the use of age-matched controls might have resulted in ovulatory cycles with inadequate progesterone secretion in this age group. This would have resulted in an inappropriate endometrial histology with a deficient secretory phase (Fox, 1995b). It is indeed well documented that LNG-IUS has a major impact on endometrium histology while the effect of age is minimal in women who have documented ovulatory cycles with adequate progesterone exposure.

Endometrial tissues

All biopsies were obtained with a Cornier Pipelle suction curette (CCD International, Paris, France) that allows the sampling of the functional layer of the endometrium. The first part of tissue was fixed in 4% formaldehyde solution in phosphate-buffered saline and embedded in paraffin for immunohistochemistry. The second part of biopsy was snap-frozen for RNA and protein extraction. Specimens obtained during the mid-secretory phase were classified according to classical histological criteria (Noyes *et al.,* 1950).

RNA and protein extraction

Total RNA was extracted with an RNeasy kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's protocol. RNA concentration was determined with an ND-1000 NanoDrop spectrophotometer (Isogen Life Science, Ilssenstein, The Netherlands). For protein extraction, samples were lysed in RIPA lysis buffer [50 mM Tris-HCI, 150 mM NaCI, % Nonidet P-40, 1 % Triton X-100, 1 % Na-deoxycholate, 0.1 % SDS; supplemented with Complete Mini proteinase inhibitor cocktail (Roche/ Boehringer, Mannheim, Germany)]. The total protein concentrations of specimens were determined with a BioRad Dc Protein Assay (BioRad, Hercules, CA, USA).

RT-PCR amplification

28S ribosomal RNA (rRNA) and MMP mRNAs were measured in 10 ng aliquots of total RNA by RT-PCR which was performed using the GeneAmp Thermostable rTth reverse transcriptase RNA PCR kit (Perkin-Elmer, Branchburg, NJ, USA) and specific pairs of primers (5 pmol each; Eurogentec, Seraing, Belgium) as described previously (Goffin et al., 2003). RT-PCR products were resolved on 10% polyacrylamide gels and analysed using a Fluor-S Multilmage (BioRad) after staining with Gelstar dye (FMC BioProduct, Rockland, ME, USA). MMP products were quantified by normalization with respect to 28S rRNA. RT-PCR was performed in duplicate for each sample. The two experiments, with 3-15% differences, were normalized.

Zymography

Gelatinolytic activities were assessed by gelatinase zymography. First, 20 µg of tissue-extracted proteins were applied to 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin (Sigma, St Louis, MO, USA). After electrophoresis, SDS was removed from the gels by two incubations in 2% Triton X-100 for 30 min. The gels were incubated overnight at 37°C in 50 mM Tris-HCl (pH 7.4), 0.2M NaCl, 5 mM $CaCl_2$ and 1 µM $ZnCl_2$. The gels were then stained for 20 min with Coomassie Blue and destained for 2 h. Proteolytic activities were evidenced as clear bands against the blue background of stained gelatin.

Immunohistochemistry

Serial sections (5-µm thick) were stained with haematoxylin and eosin or used for immunohistochemical localization of MMP-1, -2, -3, -7, -9 and CD68 specific of macrophage with mouse monoclonal antibodies (Table I). A rabbit anti-human antibody was used to stain MMP-26. Paraffin sections were first incubated for 30 min at room temperature with 3% H_2O_2 in order to block the endogenous peroxidases and then boiled in 10 nmol/L citrate buffer, pH 5.7, using a microwave oven. Sections were incubated overnight at 4°C with diluted primary antibodies, and specific binding was revealed using either Envision or an appropriate secondary Ab according to Table 1. Staining was visualized using diaminoben-zidine as chromogen.

In each run, a positive menstrual endometrium was used and negative controls were performed by replacement of the primary antibody with a non-relevant antibody of the same isotype (IGg2a against proinsulin; IgG 1 against adrenocarticotropic hormone).

Two principal cell types were detected and analysed by immunostaining for MMPs: stromal cells and decidual cells. Regarding the stromal cells, there were no variations in staining intensity. Semi-quantification was made on the basis of the number of positive cells, and at least a cluster of stromal cells was present to consider the case as positive. Decidual cells were diffusely stained, but the intensity could vary. For MMP-7 and -26, epithelial staining was also present, diffuse and intense without significant variations. Polynuclear neutrophils were stained strongly with the MMP-9 antibody. All the slides were analysed by two blinded observers.

Table I: *Primary and secondary antibodies used for immunohistochemistry*

	Primary Ab	**Secondary Ab**
MMP-1	0.2 μg/ml (provided by K. Iwata, Fuji Chemical ndustries, Ltd, Toyama, Japan)	Envision system labelled HRP anti-mouse (K4001, DakoCytomation, Glostrup, Denmark), for 75 min at room temperature
MMP-2	2 μg/ml (provided by K. Iwata)	Envision system labelled HRP anti-mouse, for 75 min at room temperature
MMP-3	1 μg/ml (MAB3306, R&D System, Minneapolis, MN, USA)	Envision system labelled HRP anti-mouse, for 75 min at room temperature
MMP-7	1 μg/ml (IM40L, Calbiochem, Darmstadt, Germany)	Envision system labelled HRP anti-mouse, for 75 min at room temperature
MMP-9	0.5 μg/ml (IM37L, Calbiochem)	Envision system labelled HRP anti-mouse, for 75 min at room temperature
MMP-26	1.5 μg/ml (gift from A. Sang, Florida State University)	Swine anti-rabbit (P0217, DakoCytomation) diluted 1/ 100 for 30 min, at room temperature
CD68	Ready to use (M0876, DakoCytomation)	Goat anti-mouse Ab conjugated to biotin (E433, DakoCytomation), diluted 1/ 400 for 30 min at room temperature

Macrophage quantification

Slides were observed at x 100 magnification with an Olympus microscope (Omnilabo, Aartselaar, Belgium). Five different microscopic fields of each section were analysed and macrophage numbers were determined per square millimetre using Image J 1.37v software developed by Wayne Rasband (National Institutes of Health, Bethesda, ML, USA).

Statistical analysis

Analysis for statistical significance was evaluated with non-parametric methods. Kruskal-Wallis with Dunn's correction for multiple comparisons was applied for RT-PCR analysis. Immunostaining comparison between groups was performed using the χ test with Fisher's exact correction. Statistical significance was set at $P < 0.05$.

RESULTS

Effect of LNG-IUS on MMP mRNA expression

The mRNA expression of several MMPs has been assessed in the endometrium from short- and long-term users, as well as from control women and baseline cycles sampled during the mid-secretory phase. Control and baseline groups showed identical patterns of MMP expression (data not shown). When compared with control women, a significantly higher level of mRN As in MMP-1, -2, -3, -7, -9 and -12 was observed in short-term and in long-term LNG-IUS users (Fig. 1A-F). MMP-26 presented an inverse mRNA expression pattern, since its expression was lower in the endometrium of long-term LNG-IUS users (Fig. 1G).

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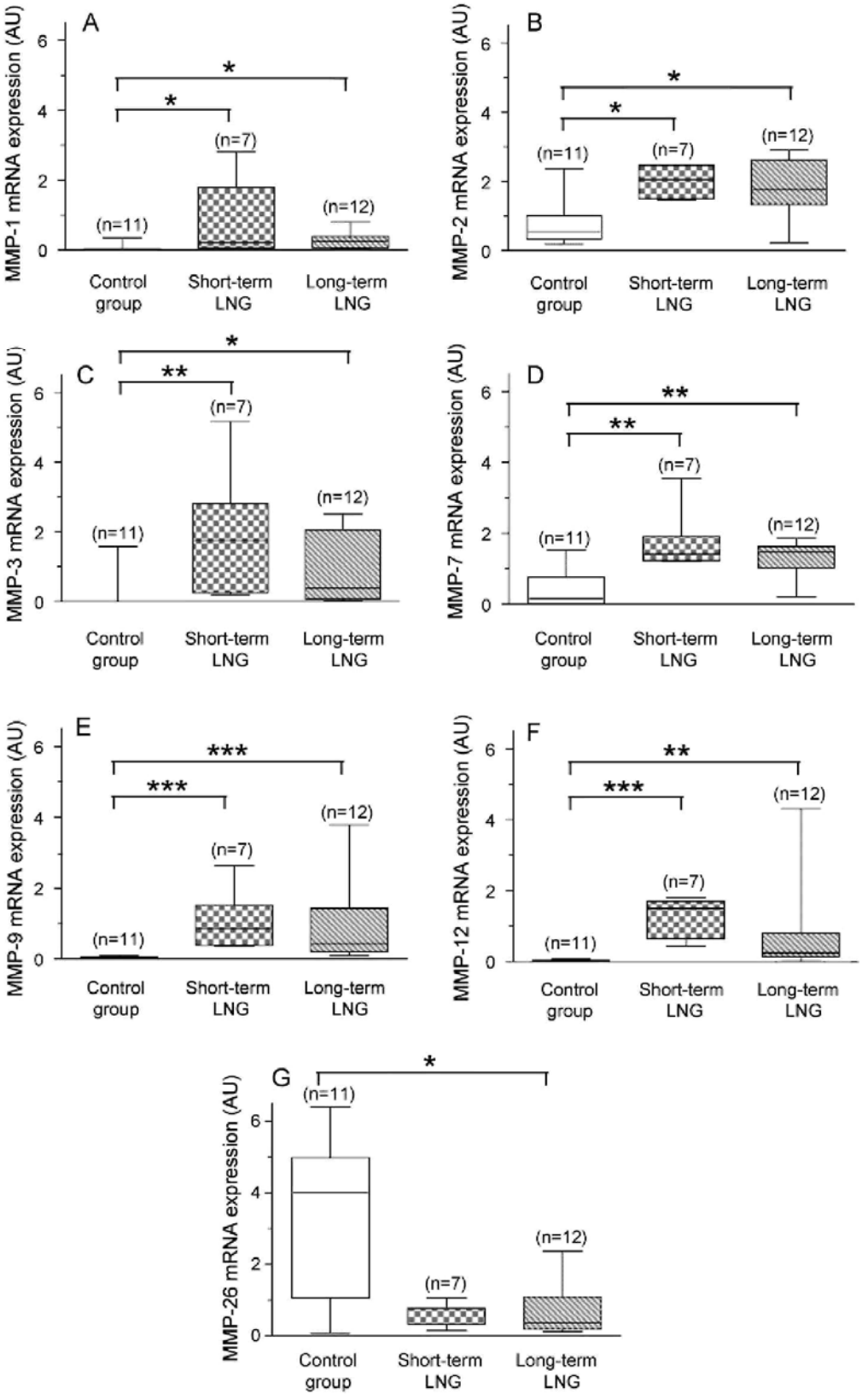
Figure 1: *Quantification of mRNA level by semi-quantitative RT-PCR. mRNA expression (mean ± SEM) of biopsies in control (n = 11), short-term LNG (n = 7) and long-term (n = 12) LNG groups. mRNA expression of MMP-1 (NbC = 30) (A), MMP-2 (NbC = 24) (B), MMP-3 (NbC = 30) (C), MMP-7 (NbC = 30) (D), MMP-9 (NbC = 30) (E), MMP-12 (NbC = 32) (F) and MMP-26 (NbC = 29) (G). The results are expressed in arbitrary units, corresponding to a ratio between the number of mRNA copies and 28S rRNA for each sample (*P ≤ 0.05; **P ≤ 0.001; ***P ≤ 0.0001). NbC, number of cycles.*



Effect of LNG-IUS on MMP-1, -3, -7 and -26 protein expressions

Exposure to LNG produced a variable picture in the endometrium, particularly in the short-term treatment group, as classically observed (Fox, 1995a). Representative illustrations are shown in Fig. 2. Most commonly, the endometrium is shallow, with somewhat limited proliferative activity and only very occasional evidence of mitosis, with few glands, variable decidualization and infiltration of macrophages plus occasional lymphocytes.

MMP-1 expression by stromal and decidual cells (Fig. 2A, B and C) was seen in all biopsies from the short-term LNG group (100%), in 66% of long-term-treated women and in 27-28% of the control or baseline groups (Table II). MMP3 was expressed by stromal cells (Fig. 2G, H and 1) in 100% of the short-term LNG group, 41% in the long-term biopsies and 14-18% in the control or baseline group (Table II). MMP-1 and -3 were expressed in significantly more biopsies of the short-term LNG group than of the control or baseline groups ($P \leq 0.003$), whereas there was no significant difference between the long-term LNG and the control groups.

In control and baseline samples (data not shown), MMP-7 was weakly stained in surface and glandular epithelial cells (Fig. 2J, K and L; data not shown). Significantly, more short-term (100%) and long-term (75%) biopsies displayed a strong MMP-7 staining compared with control or baseline biopsies (27-28%) ($P \leq 0.02$) (Table II). In contrast, an important immunostaining of MMP-26 by epithelial cells was seen in all samples of control and baseline groups (Fig. 2P). After LNG-IUS use, staining was absent in epithelial cells, whereas decidual cells showed a faint staining in only 14 and 25% of short-term and long-term groups, respectively (Table II and Fig. 2Q and R).

Effect of LNG-IUS on MMP-2 and -9 protein expressions and activation

As assessed by immunohistochemistry, MMP-2 in stromal and decidual cells was seen in a significantly higher proportion of biopsies from LNG-treated groups than from control and baseline groups ($P \leq 0.04$) (Table II, Fig. 2D, E and F). MMP-2 was immunodetected in all short-term-treated women (n = 7), but in only one-half of the long-term subjects (n = 12) (Table II).

MMP-9 immunostaining was specifically seen in polymorphonuclear inflammatory cells apposed to the endothelial cell lining of vessels in LNG-treated patients but not in control women. Here again, MMP-9 was detected in all short-term subjects (100%) and only in two-thirds (66%) of the long-term-treated women (Fig. 2M, N and O).

Zymography revealed that active forms of MMP-9 were present in the endometrium of 1/11 (9%) biopsies of control group, in no biopsy of the baseline group (data not shown) and in 3/7 (42%) of short-term subjects. Importantly, activated forms of MMP-2 were seen in all samples (100%) of short-term LNG users, in 7/11 (63%) biopsies of long-term LNG group and in only 2/11 (18%) and 0/7 biopsies of control and baseline groups (Fig. 3).

Effect of LNG-IUS on the number of macrophages in endometrium

Qualitative (Fig. 4A, B and C) and quantitative (Fig. 4D) analysis of CD68 immunostaining showed a significantly higher number of macrophages in the short-term ($P \leq 0.001$) and long-term LNG ($P \leq 0.0001$) groups when compared with the control group. No difference was observed between the LNG groups.

DISCUSSION

LNG-IUS is an intrauterine contraceptive system also licensed for the treatment of menorrhagia. The intrauterine exposure of the endometrium to locally elevated levels of LNG results in marked endometrial atrophy and variable stromal cells decidualization, confirmed by insulin-like growth factor-binding protein 1 staining (data not shown), particularly in the long-term treatment group. As documented previously, this treatment causes a variability of appearance of the endometrium particularly after short-term treatment (Fig. 2) (Fox, 1995a). This has been ascribed to several factors. First, ovulation is not consistently inhibited in these women. Second, bleeding that occurs somewhat erratically is followed by a healing rather than a true proliferative phase. Third, the degree of decidualization may vary between samples, and area in the same endometrium. Our findings are thus in accordance with previous studies (Silverberg et al., 1986; Critchley et al., 1998; Guttinger and Critchley, 2007).

Uterine bleeding at menstruation results from MMP-mediated degradation of the ECM and basement membranes (Marbaix et al., 1996; Zhang et al., 1998; Dong et al., 2002; Salamonsen et al., 2002; Zhang and Salamonsen, 2002; Goffin et al., 2003; Salamonsen, 2003; Jabbour et al., 2006). However, the role of these proteinases in the mechanisms responsible for abnormal uterine bleeding associated with LNG-IUS has not been thoroughly assessed.

MMPs are widely recognized as key regulators of ECM turnover. MMP-1 cleaves fibrillar collagens including collagen types I and III, which are major components of endometrial interstitial ECM, thereby initiating ECM breakdown. In addition, by cleaving collagen type IV, laminin and elastin, the major components of the subendothelial basement membrane, MMP-2, -3, -7, -9 and -12 represent important potential effectors for vascular rupture. In parallel, MMP-3 is an important enzyme, as it plays a central role in establishing a cascade of MMPs activation (Salamonsen and Woolley, 1996). In this study, we compared the expression of MMPs in the endometrium of normal women with spontaneous ovulatory cycles during the luteal phase or during LNG treatment, with a particular emphasis on those MMPs that are specifically up-regulated at the time of normal menstrual bleeding (Goffin et al., 2003).

Endometrial mRNA levels of MMP-1, -2, -3, -7, -9 and -12 were significantly increased in the short- and long-term LNG groups when compared with LNG-untreated samples, whereas MMP-26 expression was down-regulated by treatment with LNG. Specific immunostaining for MMPs confirmed this differential pattern of expression.

A significantly higher proportion of short-term LNG-IUS users showed immunostaining for MMP-1, -2, -3, -7, -9, whereas only MMP-2, -7 and -9 remained elevated in the long-term LNG group when compared with the control and baseline groups.

MMP-1 was localized in the decidual cells of both LNG user groups in this study and in the stromal cells of Norplant users (subcutaneous LNG) (Vincent et *al.,* 2000). It was also increased in LNG-IUS users experiencing irregular bleeding (Milne et *al.,* 2001). In these studies, MMP-1 immunostaining was distributed in decidual/stromal cells throughout endometrium but was not confined to specific areas of tissue breakdown. On the contrary, Galant et *al.* (2000) demonstrated by *in situ* hybridization that MMP-1 mRNA was synthesized in foci of stromal breakdown in the endometrium of women using Norplant.

MMP3 mRNA and protein expressed by stromal cells were also increased in both LNG user groups. This MMP3 was shown to be particularly more prominent in the bleeding group than in the amenorrhoea group (Oliveira-Ribeiro et al., 2004). A similarly elevated expression of MMP-3 was documented in Norplant users and in menstrual controls (Marbaix et *al.,* 2000; Vincent et *al.,* 2000). Collectively these data and our demonstration of an increased focal expression of MMP-1 and -3 by stromal cells after a short period of LNG-IUS use suggest that increased MMP-1 and -3 expressions may be associated with bleeding that occurs frequently in short-term LNG users.

Endometrial expression of MMP-7 is poorly defined with a reported decreased expression during the secretory phase (Graesslin et *al.,* 2006) and an increase during menstruation (Goffin et *al.,* 2003). In this study, we found that MMP-7 mRNA level and protein localization in glandular and surface epithelial cells were enhanced in LNG-exposed women both for short and long periods.

MMP-2 and -9 expressions were documented by RT-PCR and immu-nohistochemistry. Their activity was assessed by gelatin zymography. Previous studies have localized MMP-9 in endometrial glandular and stromal cells and endothelial cells of women with an LNG-IUS (Skinner et al., 1999). In contrast, in our study, MMP-9 expression was confined to polymorphonuclear inflammatory cells, specifically neutrophils identified by CD15 immunostaining (data not shown). These cells are normally present only between days 26 and 28 of the menstrual cycle, when plasma steroids levels decrease (Salamonsen et *al.,* 2002).

The absence of neutrophils from the endometrium in the control group and in the baseline group may explain the lack of MMP-2 and -9 expressions in these women. Other studies also found that MMP-9-positive cells, neutrophils and eosinophils were abundant in endometrial biopsies from Norplant or LNG-IUS users or control menstrual biopsies (Gu et *al.,* 1995; Vincent et *al.,* 1999; Salamonsen, 2003).

In our study, (pro)-MMP-2 expression was significantly increased in decidual cells of short- and long-term LNG groups. Interestingly, as demonstrated by gelatin zymography, active MMP-2 was present in a higher proportion of women exposed to LNG-IUS.

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Figure 2: *Immunohistochemistry of MMPs. Representative negative immunostaining of MMPs in a mid-secretory phase (control group) (n = 11) (A, D, G, J and M) and positive immunostaining in a short-term LNG (n = 7) (B, E, H, K and N) and a long-term LNG (n = 12) (C, F, I, L and O) biopsy. Opposite immunostaining was observed for MMP-26 (P, Q and R). Scale bar: 100 µm.*

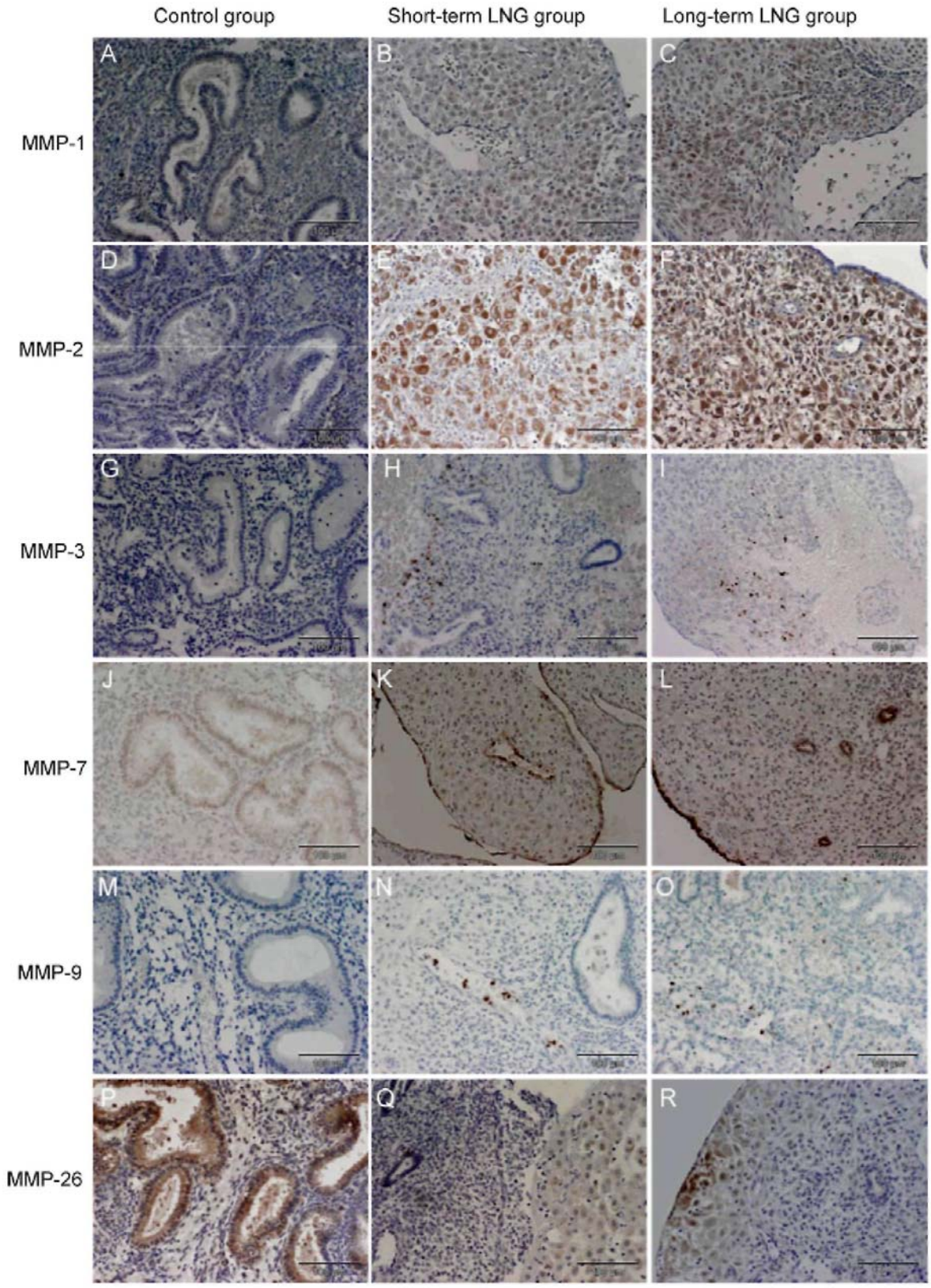

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Table II: *Incidence of positive immunostaining of MMP-1, -2, -3, -7, -9 and MMP-26 in the control, baseline, short-term and long-term LNG groups*

	Control group (*n* = 11)	Baseline group (*n* = 7)	Short-term LNG group (*n* = 7)	Long-term LNG group (*n* = 12)
MMP-1	3 (27%)	2 (28%)	7 (100%)**	8 (66%)
MMP-2	1 (9%)	1 (14%)	7 (100%)***	6 (50%)*†
MMP-3	2 (18%)	1 (14%)	7 (100%)**	5 (41%)†
MMP-7	3 (27%)	2 (28%)	7 (100%)**	9 (75%)*
MMP-9	0 (0%)	0 (0%)	7 (100%)***	8 (66%)**
MMP-26	11 (100%)	7 (100%)	1 (14%)***	3 (25%)***

*Compared with control group or with baseline group; $P < 0.05$; $P < 0.001$; $P < 0.0001$.
†Compared with short-term group LNG, †$P < 0.05$.

Figure 3: *Gelatin zymography. MMP-2 and -9 expressions in endometrial tissue extract in control (n = 11), short-term LNG (n = 7) and long-term LNG (n = 11, one protein extract was lost) groups. Gelatinase activity appears as bands of gelatin clearing. Medium conditioned by HT-1080 cells in the presence or absence of Concanavallin A (ConA) was used as a control, containing the latent 92-kDa pro-MMP-9, the latent 72-kDa pro-MMP-2 and its 62- and 59-kDa-activated forms.*

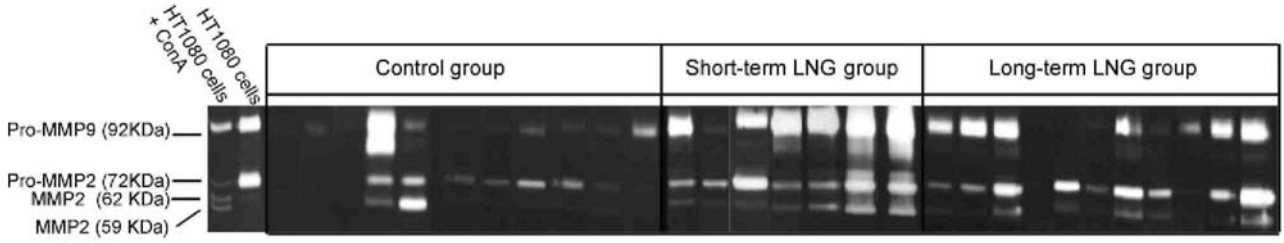
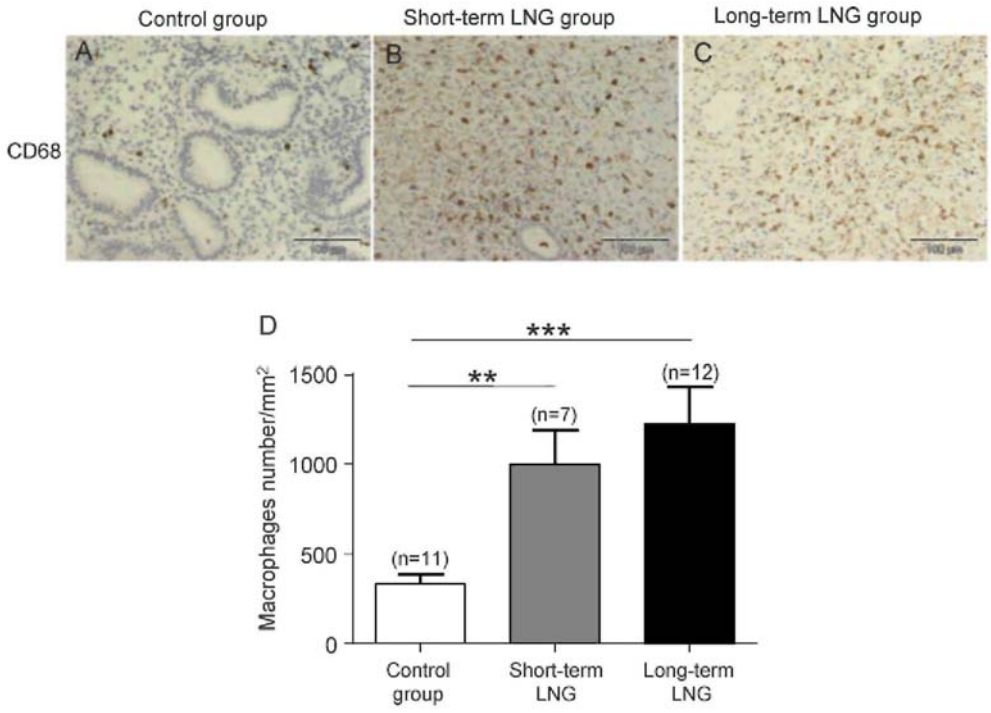


Figure 4: *Macrophage staining and quantification. Macrophage staining using CD68 Ab in control (A) (n = 11), short-term LNG (B) (n = 7) and long-term LNG (C) (n = 12) groups. Macrophage number/mm^2 (D) in the control and LNG groups (**P ≤ 0.001, ***P ≤ 0.0001). Scale bar: 100 µm.*



MMP-12 expression has also been described predominantly in the perimenstrual period, suggesting a role in tissue breakdown (Goffin et *al.,* 2003; Pilka et *al.,* 2004). By a semi-quantitative RT-PCR analysis, we demonstrate here that MMP-12 mRNA level increased significantly in short- and long-term LNG biopsies. Macrophages represent in most tissues its major source (Shapiro et *al.,* 1993). In our study, these CD68-positive cell numbers were associated with the elevated MMP-12 mRNA expression, as also suggested by Critchley et *al.* (1998). However the lack of monoclonal antibody specific to MMP12 precluded its immunolocalization in CD68-positive cells.

MMP-26 presented an expression profile inverse to that of other studied MMPs. Both the MMP-26 transcript and protein decreased in the two LNG groups when compared with control biopsies.

It is well known that irregular bleeding is more abundant during the first 6 months after LNG treatment and then decreases with time. In our study, we demonstrate that MMP-1, active MMP-2, MMP-3, MMP-7, MMP-9 and MMP-12 are more prevalent in the short-term LNG group, suggesting their important contribution to ECM breakdown and bleeding during first treatment months. The incidence of positive immunostaining showed a significant decrease (MMP-2 and -3) or non-significant trend of reduction (MMP-1, -7 and -9) (Table II) in the long-term LNG-IUS group. It might therefore be suggested that the decreased occurrence of unwanted spotting and bleeding associated with the LNG-IUS use is related to this change of MMP profiling.

In conclusion, LNG-IUS causes complex remodelling of the endometrium, with stromal cells decidualization, infiltration by neutrphils and macrophages and increased expression of a variety of MMPs in a pattern that modifies over time. The MMPs that are up-regulated at the time of menstruation and bleeding are identified in endometrial cells of women treated with LNG, more prominently during the first month of exposure. Such an increased expression may therefore be associated with the increased incidence of unwanted spotting and bleeding frequently encountered in these women. Locally delivered broad spectrum MMP inhibitors might eventually be useful to prevent such side effects.

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