# Chapter 8

# Role of progestin-induced mammary-derived growth hormone in the pathogenesis of cystic endometrial hyperplasia in the bitch

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**Submitted**

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# Abstract

Endogenous progesterone and synthetic progestins may induce hypersecretion of growth hormone (GH) of mammary origin, hyperplastic ductular changes in the mammary gland, and the development of cystic endometrial hyperplasia (CEH) in dogs. It was investigated whether progestin-induced mammary GH plays a role in the pathogenesis of CEH in the bitch. During one year, bitches with surgically excised mammary glands and healthy control bitches received 10 mg medroxyprogesterone acetate (MPA)/kg body weight at 4-week intervals. The 6-h plasma profile of GH was studied before and 3, 6, 9, and 12 months after the onset of MPA administration. Before and after MPA treatment, uterine and mammary tissues were collected for histological examination, for the immunohistochemical presence of GH, and for the expression of the genes encoding for GH, insulin-like growth factor-I (IGF-I), the GH receptor (GHR), and the progesterone receptor (PR).

In the control group, MPA administration resulted in higher basal plasma GH concentrations, lower area under the curves (AUCs) above the baseline for GH, lower GH pulse frequencies, and higher AUCs above the zero-level for GH compared with the mastectomized dogs. After MPA administration, the mammary tissue in the control dogs had differentiated into lobulo-alveolar structures and CEH was present in all uteri of both dog groups. In the MPA-exposed mammary tissue of the control dogs, GH could only be demonstrated immunohistochemically in proliferating epithelium. After treatment with MPA the dogs of both groups had immunohistochemically demonstrable GH in the cytoplasm of hyperplastic glandular uterine epithelial cells. RT-PCR analysis of the mammary gland tissue after MPA administration demonstrated a significant higher GH gene expression, and lower GHR and PR gene expression than before MPA administration. In the uterus, the expression of the gene encoding for GH was significantly increased in the mastectomized dogs, whereas in the control dogs the expression of the gene encoding for IGF-I had significantly increased with MPA administration. MPA treatment significantly down regulated PR gene expression in the uterus in both dog groups.

These results indicate that progestin-induced GH of mammary origin is not an essential component in the development of CEH in the bitch. Nevertheless, the presence of immunoreactive GH in the cytoplasm of hyperplastic glandular uterine epithelial cells of dogs with CEH suggests that GH may play a role in the pathogenesis of CEH.

# Introduction

Cystic endometrial hyperplasia (CEH) is a common disorder of the canine uterus and may result in infertility (McEntee, 1990; Arthur et al., 1996; Niskanen and Thrusfield, 1998). The condition begins with endometrial glandular hyperplasia and progresses to cystic transformation of the glands. When CEH is accompanied by inflammation and usually by bacterial infection, the disorder is called CEH-endometritis or, if the cervix is closed, pyometra (Dow, 1958; Hardy and Osborne, 1974; Sevelius et al., 1990; Schaefers-Okkens, 1996; Noakes et al., 2001). This systemic disease may result in death due to toxaemia, kidney problems and peritonitis (Arthur et al., 1989). The pathogenesis of CEH remains incompletely understood, but progestins play an important role in the development of CEH in bitches (Teunissen, 1952). Consequently, CEH is frequently seen in bitches treated repeatedly with progestins for oestrus prevention (Capel-Edwards et al., 1973; Sokolowski and Zimbelman, 1973; Goyings et al., 1977). Cystic endometrial hyperplasia may also develop spontaneously during the luteal phase of the oestrous cycle of middle-aged and elderly bitches, i.e. bitches that repeatedly have been under the influence of high concentrations of endogenous progesterone (Dow, 1958).

In dogs, endogenous progesterone or synthetic progestins such as medroxyprogesterone acetate (MPA) may induce growth hormone (GH) hypersecretion, leading to acromegalic features and insulin resistance (Eigenmann and Rijnberk, 1981; Eigenmann et al., 1983; Selman et al., 1994a). This GH excess originates in the mammary gland. Progestins induce the production of GH in foci of hyperplastic ductular epithelium (Selman et al., 1994b, van Garderen et al., 1997). The gene encoding GH in the mammary gland is identical to that in the pituitary gland (Mol et al., 1995a). Moreover, the progestin-induced mammary-derived protein is identical to pituitary GH and biologically active (Selman et al., 1994b). The progestin-induced elevations of plasma GH concentrations do not have a pulsatile secretion pattern (Watson et al., 1987), characteristic of pituitary GH secretion in healthy dogs (Kooistra et al., 2000). Additionally, the progestin-induced GH overproduction can neither be stimulated with GH-releasing hormone (GHRH), nor can it be inhibited by somatostatin, indicating autonomous secretion (Watson et al., 1987; Selman et al., 1991).

Locally produced GH probably plays a paracrine role in the progestin-induced proliferation and differentiation of mammary epithelium (Feldman et al., 1993; Mol et al., 1995a). Because of the similarity of the progestin-induced epithelial changes in both the

mammary gland and the uterus, it can be hypothesized that GH is also involved in the development of progestin-induced CEH. Although immunoreactive GH (iGH) has been found in uterine epithelial cells of progestin-treated dogs, the absence of mRNA encoding GH in uterine tissue suggests that it does not originate in the uterus (Kooistra et al., 1997). This finding refutes the hypothesis that local production of GH is involved in the pathogenesis of progestin-induced CEH. However, there is still the possibility that mammary-derived GH plays a role in the pathogenesis of CEH. To address this hypothesis, the effects of monthly injections with MPA on the development of CEH were investigated in bitches with surgically excised mammary glands and in a control group of bitches with intact mammary glands.

## Materials and methods

*Dogs*

Thirteen Beagle bitches, 3 to 9 years of age, were housed with outdoor access, fed a commercial dog food once a day, and given water *ad libitum*. The dogs were randomly assigned to two groups (mastectomized and control dogs). The mean age and body weight of the eight mastectomized dogs (5 years and 8 months and 10.5 kg, respectively) did not differ significantly from those of the five control dogs (5 years and 2 months and 9.5 kg, respectively). The dogs were accustomed to the laboratory environment and procedures such as collection of blood samples. Inspection of the vulva (swelling, discharge) was performed and serum progesterone concentration was measured on a regular basis to determine the stage of the oestrous cycle.

*Surgical procedure*

Prior to surgery food was withheld for 18h. After pre-anaesthetic medication with acepromazine (Placivet 2 %®, Codifar, Wommelgem, Belgium) and methadone (Mephenon®, Federa S.A., Brussels, Belgium) (iv), anaesthesia was induced with propofol (Diprivan 1 %®, Astra Zeneca, Brussels, Belgium) (iv) and maintained with isoflurane (Isoba®Vet, Schering-Plough Animal Health, Middlesex, England) in oxygen. Epidural anaesthesia (between lumbar vertebra L7 and sacrum) was performed in dogs that underwent mastectomy and consisted of lidocain hydrochloride (Xylocaïne 2 %®, NV Astra Zeneca, Brussels, Belgium), bupivacain hydrochloride (Marcaïne 0.5 %®, NV Astra Zeneca, Brussels, Belgium), and morphine hydrochloride (Stellorphine®, Stella, Liège, Belgium). Antibiotics (amoxicillin/clavulanic acid (Synulox®, Pfizer Animal Health, Borgo San Michele, Italy))

were administered pre- and postoperatively. Post surgical analgesia consisted of carprofen (Rimadyl®, Pfizer Animal Health, Dundee, Scotland, UK) and buprenorphine (Temgesic®, Schering-Plough, Brussels, Belgium).

All surgical procedures were performed during anoestrus. The tip of the right uterine horn, the corresponding ovary and the entire mammary gland were excised in eight dogs (mastectomized group). Surgical removal of all mammary gland tissue was performed in 2 sessions with an interval of 4-6 weeks. In the five control dogs only the tip of the right uterine horn and the corresponding ovary were excised.

At the end of the series of MPA injections, the remainder of the uterus and the left ovary were removed (both dog groups), and a sample of the mammary gland was collected (control dogs). In all dogs intra-uterine fluid was collected for bacteriological examination.

After surgical removal, a part of the tissue samples was fixed in a phosphate-buffered formalin solution, processed, and embedded in paraffin; another part was frozen in liquid nitrogen and stored at -80° C until analysis.

*Treatment*

Treatment was started in both groups after the first surgical procedures, i.e., during anoestrus, and consisted of subcutaneous MPA injections (Depo-Promone®, Pharmacia Animal Health, Puurs, Belgium) in a dose of 10 mg/kg body weight at intervals of 4 weeks, for a total of 13 administrations.

*Blood sample collection*

In all dogs, two blood samples, with an interval of 15 min, for determination of the plasma insulin-like growth factor-I (IGF-I) concentrations were collected by jugular venipuncture before surgery and the first MPA administration, and 1, 4, 7, and 10 months after the onset of treatment.

In all dogs, blood samples for the determination of the plasma profiles of GH were collected at 15-min intervals between 0800h and 1400h before surgery and the first MPA administration, and 3, 6, 9, and 12 months after the onset of treatment.

The blood samples were collected after an overnight fast, immediately transferred to ice-chilled EDTA-coated tubes and centrifuged at $4^{o}$ C for 10 min. Plasma was stored at $-25^{o}$ C until assayed.

*Hormone determination*

Plasma progesterone concentrations were determined with a previously validated radioimmunoassay (RIA) (Henry et al., 1987). The intra-assay and interassay coefficients of variation were 7.05 % and 8.75 %, respectively. The sensitivity of the assay was 0.005 ng.

Plasma GH concentrations were measured using a commercially available RIA for porcine and canine GH (PGH-46HK; Linco Research, St. Charles MS, USA). The intra-assay coefficient of variation was 7.6 % at a plasma concentration of 4.4 µg/l. The sensitivity of the assay was 1 µg/l.

Total plasma IGF-I concentrations were measured after acid-ethanol extraction to remove interfering IGF-binding proteins. Plasma IGF was extracted using a mixture of 87.5 % (v/v) ethanol and 12.5 % 2 M formic acid. Tubes containing 100 µl plasma and 400 µl of the ethanol-formic acid mixture were mixed thoroughly and incubated for 30 min at room temperature. After centrifugation for 30 min at 5500 g at 4° C, a 50 µl aliquot of the supernatant was diluted 1:50 with assay buffer containing 63 mM $Na_2HPO_4$ (pH 7.4), 13 mM $Na_2EDTA$, and 0.25 % (w/v) BSA. The extraction efficiency amounted to 92.5 ± 5.7 %. Plasma IGF-I concentrations were measured in a heterologous RIA validated for the dog (Favier et al., 2001). The intra-assay coefficient of variation was 8.6 % at a plasma concentration of 100 µg/l. The sensitivity of the assay was 10 µg/l. IGF-I antiserum AFP4892898 and human IGF-I for iodination were obtained from the National Hormone and Peptide Programme (Harbor-UCLA Medical Center, Torrance CA).

*Histology and immunohistochemistry*

The formalin fixed, paraffin embedded uterine and mammary tissues were cut at 5 µm for histological and immunohistological staining. For standard histological examination the slides were stained with haematoxylin and eosin (HE). Periodic Acid Shiff (PAS) stainings were made of all uteri with CEH (i.e. after treatment with MPA) and were used to assess the secretory activity of the endometrial glands.

For immunohistochemistry, a polyclonal rabbit anti-porcine GH antibody (generous gift of S.J. Dieleman, Department of Herd Health and Reproduction, Faculty of Veterinary Medicine, Utrecht University) was used in an indirect immunoperoxidase staining procedure (PAP method) as follows. The paraffin wax of the sections was removed with xylene and the sections were cleared in 100 % alcohol. Endogenous peroxidase was blocked by incubation in 1 % $H_2O_2$ in methanol for 20 min at room temperature. The sections were rehydrated by passage through 96 % and 70 % alcohol to distilled water. They were then rinsed in

phosphate-buffered saline (PBS) (3 x 5 min) and preincubated with normal goat serum in PBS (1:20) for 20 min at room temperature. Incubation with the polyclonal rabbit anti-porcine GH antibody in a 1:5000 dilution in PBS took place overnight at 4° C and then for 30 min at room temperature. After incubation, the sections were rinsed for 3 x 5 min in PBS and incubated with goat anti-rabbit serum (Dakopatts Inc., Glostrup) diluted 1:20 for 60 min. Thereafter, the sections were washed in PBS for 3 x 5 min and incubated with rabbit peroxidase-antiperoxidase complex (Dakopatts Inc., dilution 1:100). Immunoreactive GH was visualized using 0.3 % (v/v) $H_2O_2$ and 0.5 % (w/v) 3,3,-diaminobenzidine tetrahydrochloride (Sigma, Brussels, Belgium) diluted in 0.05 mol Tris/l in HCl buffer, during a 10 min incubation step. After rinsing in distilled water for 2 min the sections were counterstained with Mayer's haematoxylin for 1 min. A canine pituitary gland served as a positive control. Approximately 70 % of the cells in the adenohypophysis stained positive for GH. The negative controls were sections of uterine and mammary tissue processed in the same way, except that normal rabbit antiserum was used instead of rabbit anti-porcine GH antibody.

*Isolation of total RNA and cDNA synthesis*

Surgically removed mammary and uterine specimens were snap frozen in liquid nitrogen and stored at -80° C until used for RNA isolation. Total RNA was extracted from 0.2 to 1 g tissue using TRIzol reagent (Invitrogen, Groningen, The Netherlands). Purification was performed with the Rneasy Midi Kit (Qiagen, Leusden, The Netherlands) according to the manufacturer's protocol. Purified total RNA was subjected to DNase treatment using the DNA free kit (Qiagen, Leusden, The Netherlands). cDNA synthesis was carried out from 1.5 µg total RNA in 60 µl reaction mix using the iScript$^{tm}$ cDNA synthesis kit (Biorad, Veenendaal, The Netherlands) according to the manufacturer's protocol.

*Real time polymerase chain reaction (RT-PCR)*

Primers (Table 1) were designed using primer select software of DNA star and primer3 (Rozen and Skaletsky, 2000) according to the parameters outlined in the Biorad icycler manual. Specificity of each primer pair was confirmed by sequencing its product. HPRT and GAPDH genes were used as the non-regulated reference genes for normalization of target gene expressions.

RT-PCR was performed using the Biorad MyiQ detection system (Biorad Laboratories Ltd.) with SYBR green fluorophore. Reactions were performed in a total volume of 25 µl

containing 12.5 µl 2x SYBR green super mix (Biorad Laboratories Ltd.), 1 µl of each primer at 400 nM concentration, 0.8 µl of cDNA, and 9.7 µl RNase and DNase free water.

RT-PCR reactions for each primer set were optimized by performing reactions under a gradient of annealing temperature using five serial 10x dilutions of pooled cDNA from all tissue samples. The protocol used was as follows: denaturation (95° C for 5 min), amplification cycle repeated 40 times (95° C for 30 sec, a PCR specific annealing temperature (Table 1) for 30 sec, 72° C for 30 sec). A melt cure analysis was performed following every run to ensure a single amplified product for every reaction.

All reactions were performed in triplicate for every sample. The reference standard dilution series was repeated on every plate. Triplicate negative controls were run with every experimental plate to assess the specificity and to identify any potential contamination.

**Table 1.** Primers were designed using primer select software of DNA star and primer3 (Rozen and Skaletsky, 2000) according to the parameters outlined in the Biorad icycler manual.

| Target gene | Forward primer | Reverse primer | Optimum annealing temperature (°C) |
|---|---|---|---|
| HPRT | agcttgctggtgaaaaggac | ttatagtcaagggcatatcc | 56 |
| GH | ctgctgctcatccagtcgt | caggtccttgagcttctcgt | 60 |
| GHR | gcgcatcccagagtctaca | accatgacgaaccccatct | 58 |
| IGF-I | tgtcctcctcgcatctctt | gtctccgcacacgaactg | 60 |
| PR | caatggaagggcagcataac | cagcactttctaaggcgaca | 58 |
| GAPDH | tgtccccacccccaatgtatc | ctccgatgcctgcttcactacctt | 58 |

*Statistical analysis*

The 6-h plasma profiles of GH were analyzed by means of the Pulsar programme developed by Merriam and Wachter (1982). The programme identifies secretory peaks by height and duration from a smoothed baseline, using the assay standard deviation (SD) as a scale factor. The cut-off parameters G1-G5 of the Pulsar programme were set at 3.98, 2.40, 1.68, 1.24, and 0.93 times the assay SD as criteria for accepting peaks 1, 2, 3, 4, and 5 points wide, respectively. The smoothing time, a window used to calculate a running mean value omitting peaks, was set at 5h. The splitting cut-off parameter was set at 0.5 and the weight assigned to peaks was 0.05. The A-, B-, and C- values of the Pulsar programme, used to calculate the variance of the assay, were set at A=0, B=7.2, and C=5. The values extracted from the Pulsar analysis included the mean of the smoothed baseline, the pulse frequency, and the area under the curve (AUC). The AUC was calculated above the zero-level ($AUC_0$) as well as above the baseline ($AUC_{base}$).

The $AUC_0$ for GH, the basal plasma GH concentration and the plasma IGF-I concentration were analyzed by a mixed model with dog as random effect and time, group (mastectomized and control dog group) and their interaction as categorical fixed effects. Using this model, the mastectomized and control dog group were compared. Additionally the evolution over time of these parameters was evaluated in each group separately.

Because the GH pulse frequency and the $AUC_{base}$ for GH were not distributed normally, the two dog groups were compared at each time point (before surgery and MPA injection and 3, 6, 9, and 12 months after the onset of MPA treatment) based on the Mann-Whitney test. Furthermore, the differences within a dog group between 0 months (= before surgery and MPA treatment) and 3, 6, 9, and 12 months after the onset of MPA administration were also analyzed by the Mann-Whitney test.

All statistical tests were performed at a global 5 % significance level, applying the Bonferroni correction for multiple comparisons. All values are expressed as mean ± SEM except for the $AUC_{base}$ for GH and the GH pulse frequency which are expressed as median values. Analyses were performed with SAS version 9.1 for Windows (Insightful Corp., Seattle, US).

The difference in expression of the target genes by RT-PCR between dogs before and after treatment with MPA were assessed using the pair wise fixed reallocation randomization test incorporated in the software programme REST-XL (Pfaffl et al., 2002) at the 5 % significance level. Dogs which were subjected to mastectomy before treatment with MPA and dogs with intact mammary gland tissue were treated as separate groups for data analysis.

*Ethics of the study*

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

# Results

In both dog groups, the 6-h plasma profile of GH before surgery and MPA treatment was characterized by a fluctuating baseline with occasional distinct elevations, indicating pulsatile secretion of GH. During treatment with MPA, in the control dogs the basal secretion of GH increased and the pulsatile secretion of GH decreased, whereas in the mastecomized dogs the pulsatile secretion persisted, without changes in the basal GH concentration (Figure 1).

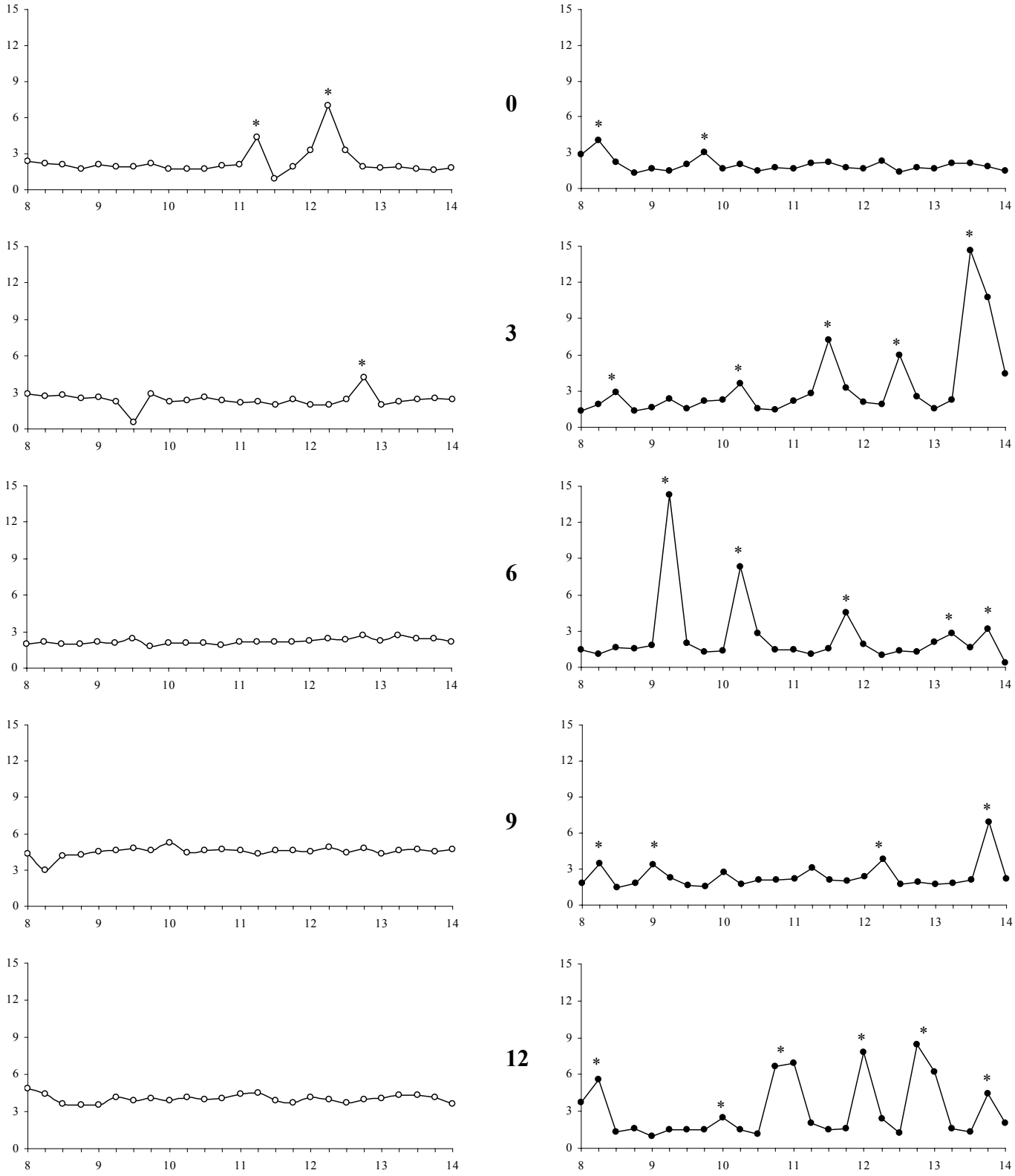


**Figure 1.** The secretory profiles of GH (µg/l) (=Y-axis) in a five-year-old mastectomized bitch (●) and in a five-year-old control bitch (○). Blood samples were collected at 15-min intervals for 6 h (from 0800h to 1400h = X-axis), before surgery and treatment with MPA (0 months) and 3, 6, 9, and 12 months after the onset of MPA treatment. Significant pulses, calculated by the Pulsar programme, are indicated by an asterisk.

The mean basal plasma GH concentration evolved significantly (P = 0.0002) different over time between both dog groups. In the control group the mean basal plasma GH concentration was significantly higher after 9 months (3.4 ± 0.5 µg/l, P < 0.0001) and 12 months (2.8 ± 0.6 µg/l, P = 0.001) of MPA administration than in the mastectomized group (1.4 ± 0.2 µg/l and 1.6 ± 0.2 µg/l, respectively). Within the mastectomized group the mean basal plasma GH concentration before surgery and treatment with MPA (1.6 ± 0.1 µg/l) did not differ significantly from that after 3 (1.9 ± 0.2 µg/l), 6 (1.1 ± 0.1 µg/l), 9 (1.4 ± 0.2 µg/l), and 12 (1.6 ± 0.2 µg/l) months of MPA treatment. Within the control group the mean basal plasma GH concentration before surgery and treatment with MPA (1.7 ± 0.1 µg/l) was significantly lower than that after 9 months (P < 0.0001) and 12 months (P = 0.0002) of MPA treatment (Figure 2a).

The mean $AUC_0$ for GH evolved significantly (P = 0.0002) different over time between both dog groups. In the control group the mean $AUC_0$ for GH after 9 months of MPA treatment (20.4 ± 2.9 µg/lx6h) was significantly higher (P = 0.0002) than that in the mastectomized group (9.5 ± 1.5 µg/lx6h). Within the mastectomized group the mean $AUC_0$ for GH before surgery and treatment with MPA (11.4 ± 0.4 µg/lx6h) did not differ significantly from that after 3 (14.2 ± 1.5 µg/lx6h), 6 (9.8 ± 1.9 µg/lx6h), 9 (9.5 ± 1.5 µg/lx6h), and 12 (11.9 ± 1.3 µg/lx6h) months of MPA treatment. Within the control group the mean $AUC_0$ for GH before surgery and treatment with MPA (11.3 ± 1.0 µg/lx6h) was significantly lower compared to that after 9 months (20.4 ± 2.9 µg/lx6h, P < 0.0001) and 12 months (17.0 ± 3.6 µg/lx6h, P = 0.005) of MPA treatment (Figure 2b).

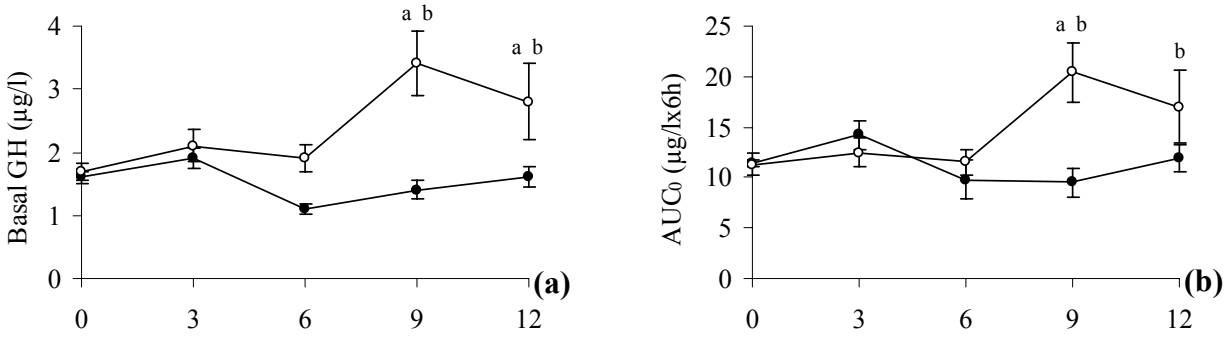


**Figure 2. (a)** The mean (± SEM) basal plasma GH concentration, **(b)** the mean (± SEM) area under the curve above the zero-level ($AUC_0$) for GH in eight mastectomized Beagle bitches (●) and in five control Beagle bitches (○). Blood samples were collected at 15-min intervals for 6 h before surgery and treatment with MPA (0 months) and 3, 6, 9, and 12 months after the onset of MPA treatment (= X-axis). 'a' indicates significant difference between both dog groups and 'b' indicates significant difference within the group compared with the value before surgery and MPA treatment.

The median $AUC_{base}$ for GH in the mastectomized group was significantly higher after 3 months (2.3 µg/lx6h; range = 0 to 9.5 µg/lx6h, P = 0.01) and 9 months (0.2 µg/lx6h; range = 0 to 5.5 µg/lx6h, P = 0.003) of MPA administration than in the control group (at both time points median $AUC_{base}$ = 0 and range = 0 to 0). In both groups, the median $AUC_{base}$ for GH before surgery and MPA treatment did not differ significantly with that after 3, 6, 9, and 12 months of MPA treatment.

The median GH pulse frequency in the mastectomized group was significantly higher after 6 months (2 peaks per 6h; range = 0 to 5 peaks per 6h, P = 0.01) and 9 months (1 peak per 6h; range = 0 to 5 peaks per 6h, P = 0.01) of MPA treatment than that in the control group (at both time points median GH pulse frequency = 0 peaks and range = 0 to 0 peaks per 6h). In both groups the median GH pulse frequency before surgery and MPA treatment did not differ significantly with that after 3, 6, 9, and 12 months of MPA treatment.

The mean plasma IGF-I concentration evolved significantly (P < 0.0001) different over time between both dog groups. In the control group, the mean plasma IGF-I concentration was significantly higher after 4 (116 ± 18 µg/l, P = 0.006), 7 (143 ± 29 µg/l, P = 0.001) and 10 months (180 ± 30 µg/l, P < 0.0001) of MPA treatment than in the mastectomized group (54 ± 9 µg/l, 68 ± 12 µg/l, and 57 ± 8 µg/l, respectively). Within the mastectomized group the mean plasma IGF-I concentration before surgery and treatment with MPA (35 ± 7 µg/l) did not differ significantly from that after 1 (35 ± 7 µg/l), 4, 7, and 10 months of MPA administration. Within the control dog group the mean plasma IGF-I concentration before surgery and treatment with MPA (28 ± 6 µg/l) was significantly lower than that after 4, 7, and 10 months of MPA treatment (P < 0.0001 at all time points) (Figure 3).

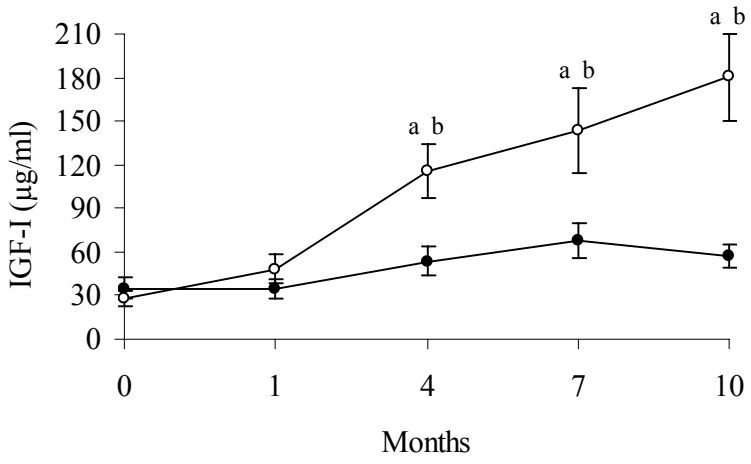


**Figure 3.** The mean (± SEM) plasma IGF-I concentration in eight mastectomized Beagle bitches (●) and in five control Beagle bitches (○) before surgery and treatment with MPA (0 months) and 1, 4, 7, and 10 months after the onset of MPA treatment. 'a' indicates significant difference between both dog groups and 'b' indicates significant difference within the group compared with the value before surgery and MPA treatment.

Histological examination of the mammary gland tissue before MPA treatment (Figure 4a) revealed inactive mammary tissue characterized by the presence of involuted ductular structures outlined by flattened epithelial cells, absent or small alveolar lumina, and a high stroma/parenchyma ratio. After MPA treatment (Figure 4b), the mammary tissue had differentiated into lobulo-alveolar structures in which milk protein synthesis occurred. The epithelial cells were cuboidal and surrounded by myoepithelial cells, and the stroma/parenchyma ratio was low. In one dog there was proliferating mammary epithelium, with focal hyperplastic changes of ductular epithelial cells, i.e. ductal budding structures. In the mammary tissues before treatment with MPA, immunohistochemical examination did not reveal iGH. Also after MPA treatment no iGH was found in the mammary tissues, except for the dog with focal hyperplastic changes of ductular epithelial cells (Figure 5a). In this dog, iGH was observed in hyperplastic ductular epithelial cells (Figure 5b).

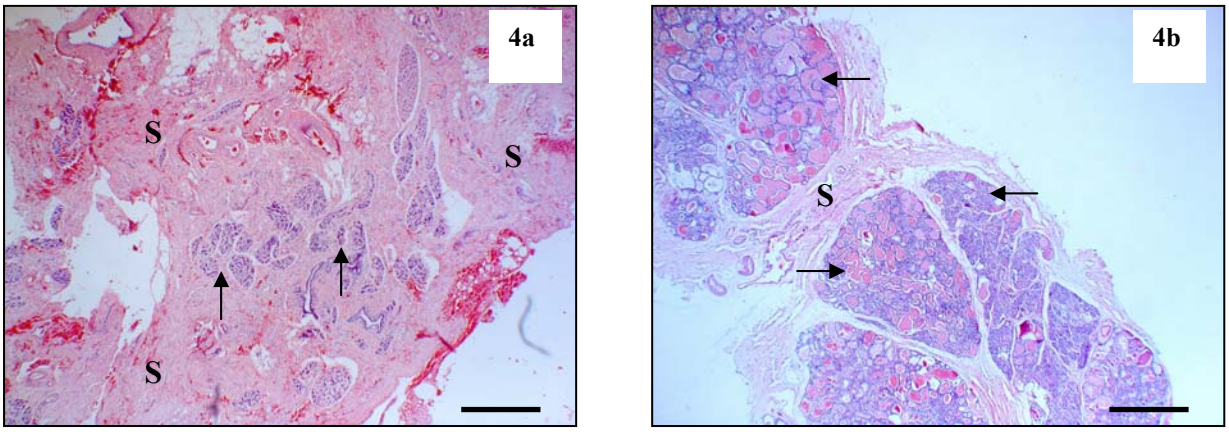


**Figure 4. (a)** Histology of mammary gland tissue before MPA treatment revealed inactive mammary tissue characterized by the presence of involuted ductular structures with only remnants of lobules (arrows) and an overrepresentation of stromal cells (S). **(b)** Histology after one year of MPA treatment revealed differentiated lobulo-alveolar glandular mammary tissue with milk protein synthesis (arrows). The amount of stroma is strongly reduced (S). HE staining - Bar = 200 µm

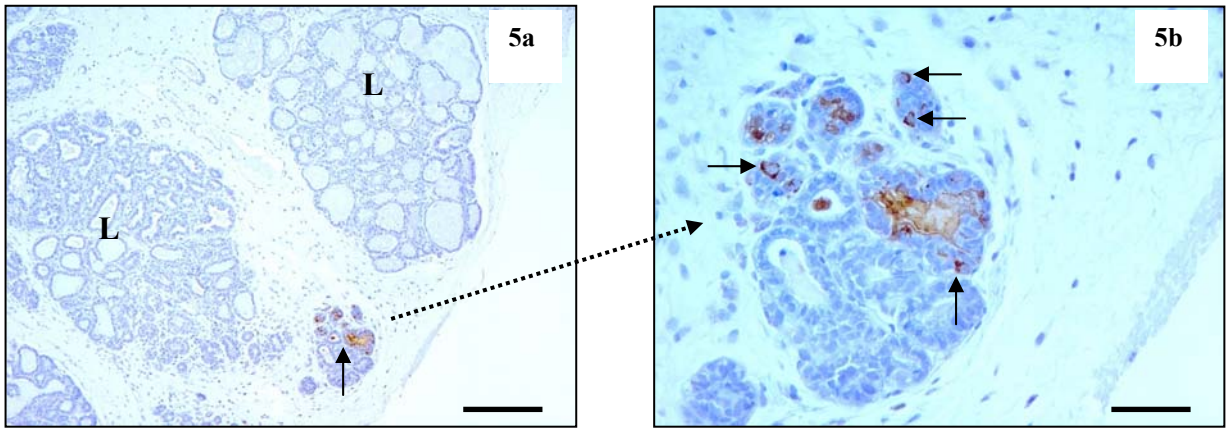


**Figure 5. (a)** Immunohistochemical examination of mammary gland tissue in a four-year-old Beagle dog after one year of MPA treatment. Immunoreactive GH is absent in lobulo-alveolar structures (L) and is focally present in hyperplastic ductular epithelial cells ("ductal buds") (arrow). Bar = 100 µm. **(b)** Enlargement of a part of figure 5a (dotted arrow). Immunohistochemical localization of GH in the cytoplasm of hyperplastic epithelial cells of mammary gland tissue after one year of treatment with MPA (arrows). Bar = 25 µm.

Histological examination of the uterine tissue before treatment with MPA confirmed that all dogs were in the anoestrous phase of the ovarian cycle and that none of the uteri showed histological signs of CEH (Figure 6a). The inactive endometrium contained small endometrial glands outlined by cuboidal cells. The myometrium consisted of smooth muscle cells with dense nuclei and a small amount of cytoplasm. After MPA administration, CEH was macro- and microscopically present in all dogs of both dog groups. Histological changes were severe and included multiple, large, mucus-filled cystic, PAS-positive endometrial glands, hyperplasia of the epithelium of the endometrial glands, labyrinth-like proliferations of the surface epithelium and endometrial stroma (Figure 6b). In all dogs, there was a variable degree of necrosis in the endometrium characterized by dense, small nuclei indicating pyknosis, and a small to moderate infiltration with inflammatory cells (neutrophils and foci of lymphocytes and plasma cells). Bacteriological examination of the intra-uterine fluid was negative in all dogs. Immunohistochemical examination of the uterine tissues before MPA treatment did not demonstrate the presence of iGH in any dog. Immunohistochemical examination of the uterine tissues after MPA treatment revealed iGH in all dogs of both dog groups. In general, iGH was located in the cytoplasm of hyperplastic glandular epithelial cells (Figures 7a and b).

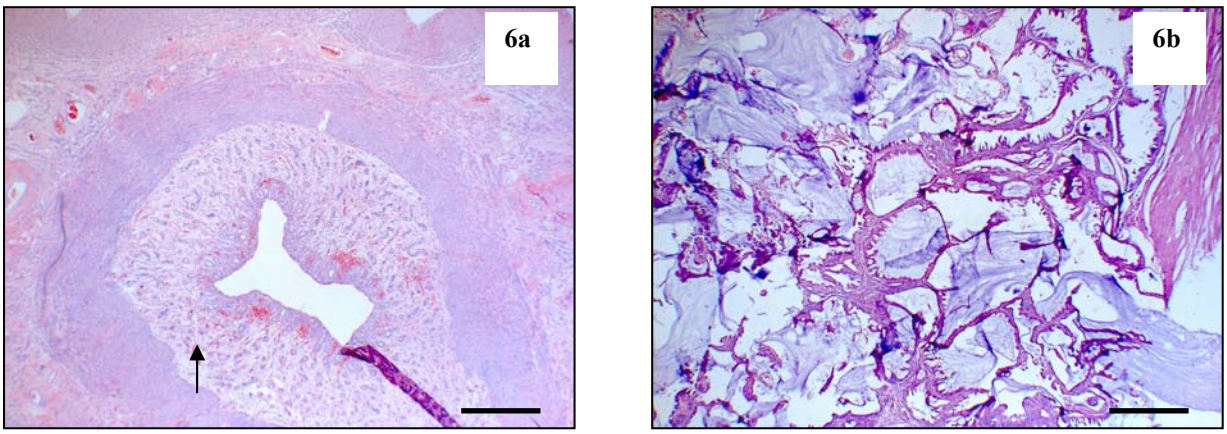


**Figure 6. (a)** Histology of the uterus before MPA administration with the characteristics of anoestrus, including the endometrium containing small endometrial glands (arrow). **(b)** Severe CEH after MPA administration characterized by multiple, large mucus-filled endometrial cysts and labyrinth-like proliferations of the surface epithelium. HE staining - Bar = 200 µm

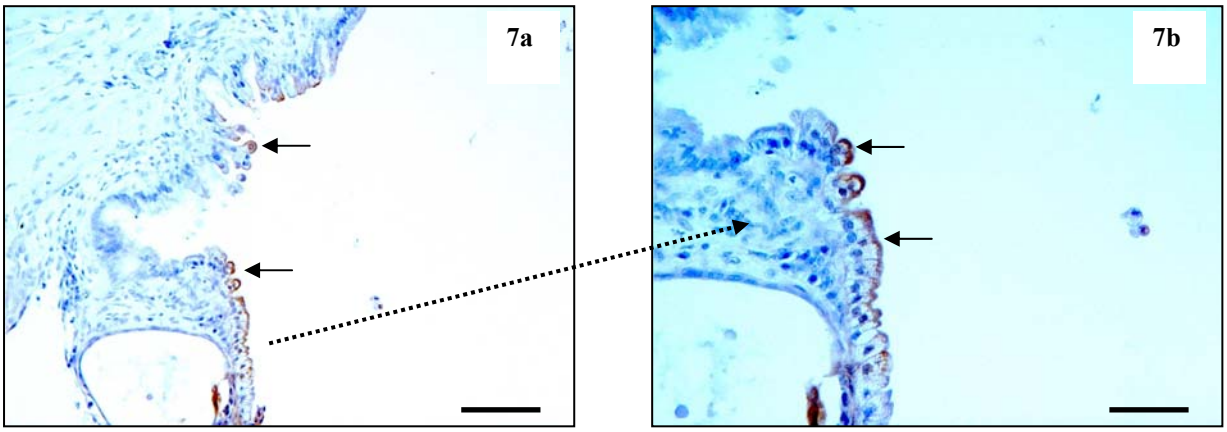


**Figure 7. (a)** Immunohistochemical examination of the uterus after one year of MPA administration shows the presence of GH in hyperplastic glandular epithelial cells (arrows). Bar = 100 µm. **(b)** Enlargement of a part of figure 7a (dotted arrow). Immunoreactive GH in the uterus is present in the vacuolated cytoplasm of the hyperplastic epithelium (arrows). Bar = 25 µm.

RT-PCR analysis of the mammary gland tissue of the control dogs after MPA treatment demonstrated a significantly (P = 0.002) higher mean GH gene expression than before treatment. The mean mammary GH receptor (GHR) and progesterone receptor (PR) gene expression was significantly (P = 0.01 and P = 0.03, respectively) lower after MPA administration than before treatment. No significant difference was observed in the mean mammary IGF-I mRNA content after MPA treatment compared to that before treatment (Figure 8). In the mastectomized dogs, the mean GH gene expression in the uterus was significantly (P = 0.002) higher after treatment with MPA than before treatment, whereas in the control dogs, the mean IGF-I gene expression was significantly (P = 0.001) higher after MPA administration. A trend (P = 0.052) for a lower mean GHR gene expression after treatment with MPA was found in the uterus of the control dogs compared with that before MPA administration. In both dog groups the mean mRNA content encoding the PR after treatment with MPA was significantly (P = 0.001 for both groups) down regulated compared to the PR mRNA content before treatment (Figure 9).

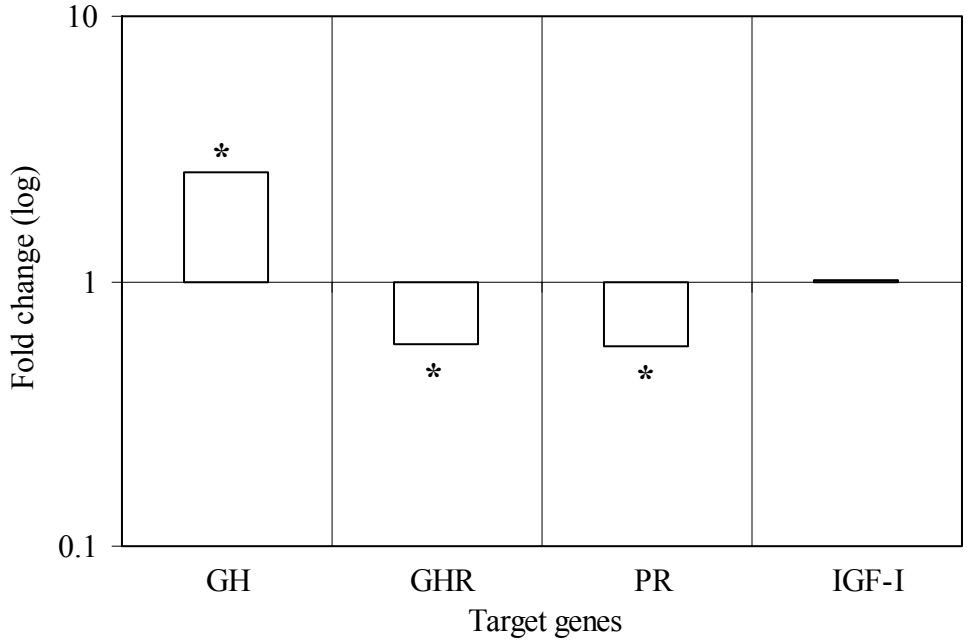


**Figure 8.** Difference in mean gene expression of growth hormone (GH), the GH receptor (GHR), the progesterone receptor (PR), and insulin like growth factor-I (IGF-I) between before and after treatment with MPA in the mammary gland of Beagle dogs demonstrated by RT-PCR. Significant differences in gene expression before and after MPA treatment are indicated with an asterisk.

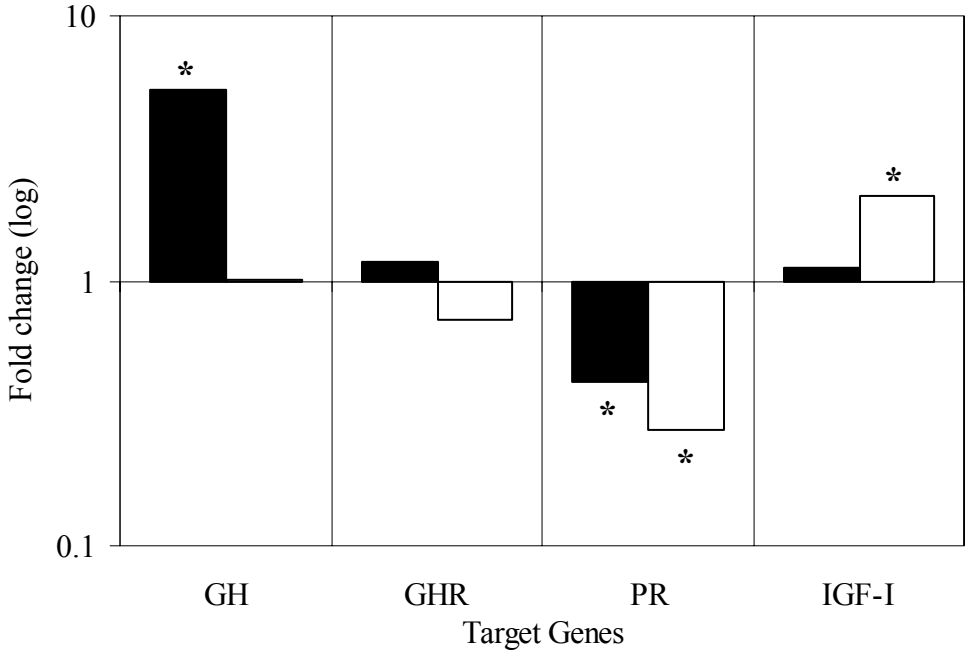


**Figure 9.** Difference in mean gene expression of growth hormone (GH), the GH receptor (GHR), the progesterone receptor (PR), and insulin like growth factor-I (IGF-I) between before and after treatment with MPA in the uterus of five control Beagle dogs (□) and eight mastectomized dogs (■) demonstrated by RT-PCR. Significant differences in gene expression before and after MPA treatment are indicated with an asterisk.

# Discussion

In the control dogs MPA treatment resulted in a higher basal GH secretion and less GH secreted in pulses compared to the mastectomized dogs. This is consistent with (partial) suppression of pituitary GH secretion by progestin-induced GH secretion from the mammary gland. In the mastectomized dogs MPA treatment did not result in a change of the basal GH concentration, the $AUC_0$ for GH, the $AUC_{base}$ for GH, and the GH pulse frequency, also demonstrating the mammary origin of progestin-induced GH excess. Dogs are not unique among other mammalian species in that the gene encoding GH is expressed in the mammary gland. RT-PCR has revealed that this gene is also expressed in the mammary gland of women and cats (Mol et al., 1995a and 1995b). However, until now the dog is the only species in which it has been demonstrated unequivocally that progestin-induced mammary-derived GH reaches the systemic circulation, and is biologically active (Selman et al., 1994b).

After several months of MPA treatment, there was a low GH pulse frequency and a corresponding low $AUC_{base}$ for GH in the control dogs compared to the mastectomized dogs.

A similarly decreased GH pulsatility has also been reported in women during the second half of pregnancy (Eriksson et al., 1989). In these women the loss of GH pulsatility is due to the release of a placental GH variant (Eriksson et al., 1989). Thus, in both species the loss of GH pulsatility can be ascribed to the negative feedback effects of non-episodically secreted extra-pituitary GH. It has been demonstrated in humans that GH exerts its negative feedback by a stimulation of hypothalamic somatostatin secretion (Berelowitz et al., 1981). Additionally, in the control dogs the progestin-induced elevated GH concentrations had induced raised IGF-I concentrations, which will have contributed to the inhibition of pulsatile pituitary GH secretion (Hartman et al., 1993).

With regard to the physiological role of the progestin-induced mammary GH production, local autocrine and paracrine effects in the mammary gland as well as systemic endocrine effects have to be considered. It is thought that the progestin-induced GH production in the mammary gland leads to local production of IGFs, whereby the growth-promoting effect is modulated by locally synthesized IGF-binding proteins (Mol et al., 1996). Thus a proliferative environment for the glandular epithelium is created, i.e., the autocrine/paracrine background for the physiological proliferation and differentiation of mammary gland tissue. This is in agreement with the results of studies in rats, indicating that GH and GH-induced IGF-I are necessary for mammary development (Feldman et al., 1993; Walden et al., 1998). Also in the ewe mammogenesis is partly dependent upon GH (Kann, 1997). However, until now it has not been demonstrated in this species that this GH originates from the mammary gland.

In the dog, the histological features of the mammary gland vary strongly with the stage of the ovarian cycle (van Garderen et al., 1999). In the anoestrous phase, the glandular tissue is inactive, and microscopically only involuted ductular structures and remnants of lobules are encountered. These were also the characteristics of the mammary gland tissue of our dogs before treatment with MPA. In the luteal phase, after ovulation, there is nodular epithelial proliferation resulting in ductal buds that parallels high endogenous progesterone concentrations in dogs. A similar proliferation of these epithelial cells can be induced by exogenous progestins. In the bitch, ovulation is followed by a relatively long luteal phase, irrespective of pregnancy (Concannon et al., 1975). During this longstanding progesterone-dominated phase of approximately two months, epithelial cells in budding structures proliferate and finally differentiate into lobulo-alveolar structures, fully equipped for milk synthesis. In the present study, mammary gland tissue was examined histologically after a long period of progestin administration. This may be the reason that epithelial buds were

found in only one dog and that most of the glandular tissue had differentiated into lobulo-alveolar structures in which milk synthesis occurred.

In canine mammary tissue iGH and GH gene expression is found predominantly in the ductal epithelial buds in the early and midluteal phase of the ovarian cycle (van Garderen et al., 1997). In contrast, the GH gene expression is diminished in differentiated lobulo-alveolar glandular tissue and in the anoestrous phase of the canine ovarian cycle (van Garderen et al., 1997). Similar immunohistochemical features were found in the present study. Immunoreactive GH was not detected in the mammary gland tissue of the anoestrous dogs before treatment with MPA. Additionally, iGH was absent in the mammary gland tissue of the control bitches treated for twelve months with MPA, except for one dog. In this dog, iGH appeared to be present only in hyperplastic ductular epithelium that consisted of more than 2 cell layers, i.e. epithelial cells in budding structures.

RT-PCR analysis, which is more sensitive than immunohistochemical examination demonstrated that MPA administration increased GH gene expression in mammary gland tissue of the control dogs. Also in another study, increased GH mRNA levels were found in mammary gland tissue of dogs after prolonged treatment with progestins (Mol et al., 1995a). Whereas in primates, GH can bind to the GHR and the prolactin receptor, in nonprimate mammals, such as the dog, GH can only bind to its specific receptor, the GHR (Rutteman et al., 1986). van Garderen et al. (1999) demonstrated that immunohistochemical expression of the GHR is down regulated in completely differentiated alveolar epithelial cells at the end of the luteal phase. Similarly, RT-PCR analysis of the mammary tissue in our control dogs after prolonged MPA treatment confirmed that GHR gene expression was significantly lower compared to this expression before MPA administration.

A possible systemic endocrine effect of mammary GH is the effect on uterine epithelium. After MPA treatment the uterine tissues of both the intact and the mastectomized dogs had iGH. This indicates that progestin-induced mammary-derived GH does not play a key role in the development of CEH in the bitch. Nevertheless, the widespread presence of iGH in uterine epithelial cells of dogs with CEH suggests an association between GH and this uterine pathology. Next to GH, a previous study (De Cock et al., 2000) also demonstrated the presence of iIGF-I in the uterus of dogs with CEH. These growth factors have an intrinsic growth promoting effect on most cell types in the body (Schoenle et al., 1982; Simmen, 1991; Jones and Clemmons, 1995; McCusker, 1998). The growth promoting effect of IGF-I on the uterus is further illustrated by the fact that IGF-I knock out mice have severe hypoplastic uteri (Baker et al., 1996). In addition, IGF-I is involved in the postnatal growth of the uterus in the

rat (Gu et al., 1999) and the pig (Simmen et al., 1992). IGF-I treatment of ovariectomized rats induces uterine endometrial hyperplasia (Sahlin et al., 1994).

RT-PCR revealed that, after MPA treatment, the GH mRNA content of uterine tissue was only increased in the mastectomized dogs and not in the control dogs. Comparable with the progestin-induced GH gene expression in canine mammary tissue during development of ductal epithelial buds (van Garderen et al., 1997), MPA treatment also resulted in more GH gene expression in the uterine epithelial tissue. Apparently, in the control dogs the elevated circulating concentrations of GH of mammary origin and the subsequently increased plasma IGF-I concentrations suppressed uterine GH gene expression, similar to what has been reported for the pituitary (Hartman et al., 1993). MPA treatment also resulted in increased expression of the IGF-I gene in the uterine tissue, but this was significant only in the control dogs. This may be explained by the stimulating effect of the elevated circulating concentrations of GH, originating from the mammary gland, on uterine IGF-I gene expression in these dogs. As the presence of mRNA encoding for the GHR has already been demonstrated in the human uterus (Mercado et al., 1994; Sharara and Nieman, 1995), circulating GH may indeed influence intracellular effects in uterine cells.

In this study MPA did not promote the expression of GHRs in uterine epithelium. This makes it unlikely that increased numbers of GHRs can explain the presence of iGH in uterine cells, as proposed earlier (Kooistra et al., 1997).

Uterine steroid hormone receptor status is a crucial element in pathological conditions such as CEH and pyometra (De Cock et al., 1997; Noakes et al., 2001). Progesterone exposure is considered the initiating step in the development of CEH (Teunissen, 1952; Capel-Edwards et al., 1973; Sokolowski and Zimbelman, 1973; Goyings et al., 1977; Evans and Sutton, 1989; Allen, 1992; Von Berky and Townsend, 1993). In the present study one year of MPA treatment decreased the expression of the gene encoding for PR. In line with this observation, immunohistochemical studies (De Bosschere et al., 2002) found a severe reduction in the canine uterine PR after 12 weeks of MPA administration. The uterine concentration and distribution of progesterone receptors are influenced by hormonal changes during the oestrous cycle and by administration of MPA in the bitch (Dhaliwal, 1997). An increase in plasma progesterone concentration results in a decline of PR content in the uterus (Fernandes et al., 1989; Clarke and Sutherland, 1990; Vesanen et al., 1991; Graham and Clarke, 1997; Vermeirsch et al., 2000) and explains the decrease of PR mRNA with MPA treatment in the present study.

In conclusion, MPA administration caused hypersecretion of GH and IGF-I in the control dogs, but failed to do so in the mastectomized dogs. All dogs of both groups developed CEH, indicating that mammary GH is not a requirement for the development of progestin-induced CEH. Nevertheless, the presence of iGH in the cytoplasm of hyperplastic glandular uterine epithelial cells of dogs with CEH suggests that GH may play a role in the pathogenesis of CEH.

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## References

Allen WE (1992). Fertility and Obstetrics in the dog, pp 82-86. Blackwell Scientific Publications, Oxford.

Arthur GH, Noakes DE, Pearson H (1989). Infertility in the dog. In: Veterinary Reproduction and Obstetrics (Theriogenology), pp 496-500. Ed. G.H. Arthur. Baillière Tindall, London.

Arthur GH, Noakes DE, Pearson H, Parkinson T (1996). Infertility in the bitch and queen. In: Veterinary Reproduction and Obstetrics, pp 527-529. Ed. G.H. Arthur. WB Saunders Company Limited, London.

Baker J, Hardy MP, Zhou J, Bondy C, Lupu F, Bellve AR, Efstratiadis A. Effects of an Igf1 gene null mutation on mouse reproduction. Mol Endocrinol 1996;10:903-918.

Berelowitz M, Szabo M, Frohman LA, Firestone S, Chu L, Hintz RL. Somatomedin-C mediates growth hormone negative feedback by effects on both the hypothalamus and the pituitary. Science 1981;212:1279-1281.

Capel-Edwards K, Hall DE, Fellowes KP, Vallance DK, Davies MJ, Lamb D, Robertson WB. Long-term administration of progesterone to the female beagle dog. Toxicol Appl Pharmacol 1973;24:474-488.

Clarke CL and Sutherland RL. Progestin regulation of cellular proliferation. Endocr Rev 1990;11:266-301.

Concannon PW, Hansel W, Visek WJ. The ovarian cycle of the bitch: plasma estrogen, LH and progesterone. Biol Reprod 1975;13:112-121.

De Bosschere H, Ducatelle R, Tshamala M, Coryn M. Changes in sex hormone receptors during administration of progesterone to prevent oestrus in the bitch. Theriogenology 2002;58:1209-1217.

De Cock H, Vermeirsch H, Ducatelle R, De Schepper J. Immunohistochemical analysis of the estrogen receptor in cystic-endometritis-pyometra complex in the bitch. Theriogenology 1997;48:1035-1047.

De Cock HEV, Ducatelle R, Tilmant K, De Schepper J. Possible role for insulin-like growth factor I in the pathogenesis of cystic endometrial hyperplasia pyometra complex in the bitch. 2000, PhD thesis, Ghent University.

Dhaliwal GK. The pathogenesis of cystic endometrial hyperplasia (pyometra) in the bitch with particular reference to the role of steroid receptors. 1997, PhD thesis, University of London.

Dow C. The cystic hyperplasia-pyometra complex in the bitch. Vet Rec 1958;70:1102-1110.

Eigenmann JE, Eigenmann RY, Rijnberk A, van der Gaag I, Zapf J, Froesch ER. Progesterone-controlled growth hormone overproduction and naturally occurring canine diabetes and acromegaly. Acta Endocrinol (Copenh) 1983;104:167-176.

Eigenmann JE and Rijnberk A. Influence of medroxyprogesterone acetate (Provera) on plasma growth hormone levels and on carbohydrate metabolism. I. Studies in the ovariohysterectomized bitch. Acta Endocrinol (Copenh) 1981;98:599-602.

Eriksson L, Frankenne F, Eden S, Hennen G, Von Schoultz B. Growth hormone 24-h serum profiles during pregnancy - lack of pulsatility for the secretion of the placental variant. Br J Obstet Gynaecol 1989;96:949-953.

Evans JM and Sutton DJ. The use of hormones, especially progestagens, to control oestrus in bitches. J Reprod Fertil Suppl 1989;39:163-173.

Favier RP, Mol JA, Kooistra HS, Rijnberk A. Large body size in the dog is associated with transient growth hormone excess at a young age. J Endocrinol 2001;170: 479-484.

Feldman M, Ruan W, Cunningham BC, Wells JA, Kleinberg DL. Evidence that the growth hormone receptor mediates differentiation and development of the mammary gland. Endocrinology 1993;133:1602-1608.

Fernandes PA, Bowen RA, Sawyer HR, Nett TM, Gorell TA. Concentration of receptors for estradiol and progesterone in canine endometrium during estrus and diestrus. Am J Vet Res 1989;50:64-67.

Goyings LS, Sokolowski JH, Zimbelman RG, Geng S. Clinical, morphologic, and clinicopathologic findings in Beagles treated for two years with melengestrol acetate. Am J Vet Res 1977;38:1923-1931.

Graham JD and Clarke CL. Physiological action of progesterone in target tissues. Endocr Rev 1997;18:502-519.

Gu Y, Branham WS, Sheehan DM, Webb PJ, Moland CL, Streck RD. Tissue-specific expression of messenger ribonucleic acids for insulin-like growth factors and insulin-like growth factor-binding proteins during perinatal development of the rat uterus. Biol Reprod 1999;60:1172-1182.

Hardy RM and Osborne CA. Canine pyometra: pathophysiology, diagnosis, and treatment of uterine and extra-uterine lesions. J Am Anim Hosp Assoc 1974;10:245-268.

Hartman ML, Clayton PE, Johnson ML, Celniker A, Perlman AJ, Alberti KG, Thorner MO. A low dose euglycemic infusion of recombinant human insulin-like growth factor I rapidly suppresses fasting-enhanced pulsatile growth hormone secretion in humans. J Clin Invest 1993;91:2453-2462.

Henry M, Figueiredo AE, Palhares MS, Coryn M. Clinical and endocrine aspects of the oestrous cycle in donkeys (Equus asinus). J Reprod Fertil Suppl 1987;35:297-303.

Jones JI and Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 1995;16:3-34.

Kann G. Evidence for a mammogenic role of growth hormone in ewes: effects of growth hormone-releasing factor during artificial induction of lactation. J Anim Sci 1997;75:2541-2549.

Kooistra HS, Okkens AC, Mol JA, van Garderen E, Kirpensteijn J, Rijnberk A. Lack of association of progestin-induced cystic endometrial hyperplasia with GH gene expression in the canine uterus. J Reprod Fertil Suppl 1997;51:355-361.

Kooistra HS, den Hertog E, Okkens AC, Mol JA, Rijnberk A. Pulsatile secretion pattern of growth hormone during the luteal phase and mid-anoestrus in beagle bitches. J Reprod Fertil. 2000;119:217-222.

McCusker RH. Controlling insulin-like growth factor activity and the modulation of insulin-like growth factor binding protein and receptor binding. J Dairy Sci 1998;81:1790-1800.

McEntee K (1990). The uterus: atrophic, metaplastic, and proliferative lesions. In: Reproductive Pathology of Domestic Mammals, pp 171-174. Academic Press, San Diego.

Mercado M, DaVila N, McLeod JF, Baumann G. Distribution of growth hormone receptor messenger ribonucleic acid containing and lacking exon 3 in human tissues. J Clin Endocrinol Metab 1994;78:731-735.

Merriam GR and Wachter KW. Algorithms for the study of episodic hormone secretion. Am J Physiol 1982;243:310-318.

Mol JA, van Garderen E, Selman PJ, Wolfswinkel J, Rijnberk A, Rutteman GR. Growth hormone mRNA in mammary gland tumors of dogs and cats. J Clin Invest 1995a; 95:2028-2034.

Mol JA, Henzen-Logmans SC, Hageman P, Misdorp W, Blankenstein MA, Rijnberk A. Expression of the gene encoding growth hormone in the human mammary gland. J Clin Endocrinol Metab 1995b;80:3094-3096.

Mol JA, van Garderen E, Rutteman GR, Rijnberk A. New insights in the molecular mechanism of progestin-induced proliferation of mammary epithelium: induction of the local biosynthesis of growth hormone (GH) in the mammary glands of dogs, cats and humans. J Steroid Biochem Mol Biol 1996;57:67-71.

Niskanen M and Thrusfield MV. Associations between age, parity, hormonal therapy and breed, and pyometra in Finnish dogs. Vet Rec 1998;143:493-498.

Noakes DE, Dhaliwal GK, England GC. Cystic endometrial hyperplasia/pyometra in dogs: a review of the causes and pathogenesis. J Reprod Fertil Suppl 2001;57:395-406.

Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002;30:9 e36.

Rozen S and Skaletsky H. Primer3 Programme available at: http://www.genome.wi.mit.edu /cgi-bin/primer/primer3_www.cgi.

Rutteman GR, Willekes-Koolschijn N, Bevers MM, Van der Gugten AA, Misdorp W. Prolactin binding in benign and malignant mammary tissue of female dogs. Anticancer Res 1986;6:829-835.

Sahlin L, Norstedt G, Eriksson H. Estrogen regulation of the estrogen receptor and insulinlike growth factor-I in the rat uterus: a potential coupling between effects of estrogen and IGF-I. Steroids 1994;59:421-430.

Schaefers-Okkens AC (1996). Ovaries. In: Clinical Endocrinology of dogs and cats, pp 131-156. Ed. A. Rijnberk. Kluwer Academic Publishers, Dordrecht/Boston.

Schoenle E, Zapf J, Humbel RE, Froesch ER. Insulin-like growth factor I stimulates growth in hypophysectomized rats. Nature 1982;296:252-253.

Selman PJ, Mol JA, Rutteman GR, Rijnberk A. Progestins and growth hormone excess in the dog. Acta Endocrinol (Copenh) 1991;125:42-47.

Selman PJ, Mol JA, Rutteman GR, Rijnberk A. Progestin treatment in the dog. I. Effects on growth hormone, insulin-like growth factor I and glucose homeostasis. Eur J Endocrinol 1994a;131:413-421.

Selman PJ, Mol JA, Rutteman GR, van Garderen E, Rijnberk A. Progestin-induced growth hormone excess in the dog originates in the mammary gland. Endocrinology 1994b; 134:287-292.

Sevelius E, Tidholm A, Thoren-Tolling K. Pyometra in the dog. J Am Anim Hosp Ass 1990;26, 33-38.

Sharara FI and Nieman LK. Growth hormone receptor messenger ribonucleic acid expression in leiomyoma and surrounding myometrium. Am J Obstet Gynecol 1995;173:814-819.

Simmen FA, Simmen RC, Geisert RD, Martinat-Botte F, Bazer FW, Terqui M. Differential expression, during the estrous cycle and pre- and postimplantation conceptus development, of messenger ribonucleic acids encoding components of the pig uterine insulin-like growth factor system. Endocrinology 1992;130:1547-1556.

Simmen FA. Expression of the insulin-like growth factor-I gene and its products: complex regulation by tissue specific and hormonal factors. Domest Anim Endocrinol 1991;8:165-178.

Sokolowski JH and Zimbelman RG. Canine reproduction: effects of a single injection of medroxyprogesterone acetate on the reproductive organs of the bitch. Am J Vet Res 1973;34:1493-1499.

Teunissen GHB. The development of endometritis in the dog and the effect of oestradiol and progesterone in the uterus. Acta Endocrinologica 1952; 9:407-420.

van Garderen E, de Wit M, Voorhout WF, Rutteman GR, Mol JA, Nederbragt H, Misdorp W. Expression of growth hormone in canine mammary tissue and mammary tumors. Evidence for a potential autocrine/paracrine stimulatory loop. Am J Pathol 1997;150:1037-1047.

van Garderen E, van der Poel HJ, Swennenhuis JF, Wissink EH, Rutteman GR, Hellmen E, Mol JA, Schalken JA. Expression and molecular characterization of the growth hormone receptor in canine mammary tissue and mammary tumors. Endocrinology 1999;140:5907-5914.

Vermeirsch H, Simoens P, Hellemans A, Coryn M, Lauwers H. Immunohistochemical detection of progesterone receptors in the canine uterus and their relation to sex steroid hormone levels. Theriogenology 2000;53:773-788.

Vesanen M, Isomaa V, Alanko M, Vihko R. Bovine uterine, cervical and ovarian estrogen and progesterone receptor concentrations. Anim Reprod Sci 1991;26:61-71.

Von Berky AG and Townsend WL. The relationship between the prevalence of uterine lesions and the use of medroxyprogesterone acetate for canine population control. Aust Vet J 1993;70:249-250.

Walden PD, Ruan W, Feldman M, Kleinberg DL. Evidence that the mammary fat pad mediates the action of growth hormone in mammary gland development. Endocrinology 1998;139:659-662.

Watson ADJ, Rutteman GR, Rijnberk A, Mol JA. Effect of somatostatin analogue SMS 201-995 and antiprogestin agent RU 486 in canine acromegaly. Front Horm Res 1987;17:193-198.