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Original article

Assessment of potential clinical approaches for the expression of prolactin receptor (PRL-R) and vascular endothelial growth factor (VEGF) in various feline mammary gland tumors

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Abstract

Feline mammary gland tumors are a serious health concern, resulting in a significant reduction in the animal's lifespan and a decrease in the overall quality of life. Malignant tumors often lead to recurrences and metastases. Among endogenous factors that may influence the development or progression of mammary neoplasia, prolactin (PRL) and vascular endothelial growth factor (VEGF) appear to be of crucial importance. This study involved 60 queens with surgically removed mammary gland tumors, which were subsequently stained with hematoxylin and eosin (HE) and immunofluorescence to assess the expression of PRL and VEGF. Variables considered during analyses included the time of ovariohysterectomy, inflammation severity and clinical tumor behavior. The VEGF expression in tumors exhibited an increase in malignant cases, providing evidence of heightened angiogenesis. A lack of differences in the overall expression of PRL receptor was found between tumor types. However, the lower expression of PRL receptor in tumors with increased inflammation may suggest PRL's immunomodulating functions in feline malignant neoplastic tumors. Interestingly, the absence of positive influence of gonadectomy on tumor behavior highlights the need for further research regarding this form of prevention. High expression of PRL receptor and VEGF only in distant metastases may prompt future research on the proangiogenic function of PRL in feline mammary gland tumors.

Keywords: adenocarcinoma; adenoma; cat, mammary gland; prolactin, prolactin receptor, vascular endothelial growth factor

Introduction

For companion animal veterinary practitioners, mammary gland tumors in female cats represent a frequent and significant challenge. The most frequent histogenetic types of tumors are adenocarcinomas of various degrees of malignancy. The second most frequent tumors are adenoma-type benign lesions, dysplasia, and cystic hyperplasia. Among benign neoplastic processes, fibroadenoma, papillomas, and benign mixed tumors, are seen rather rarely (Viste et al. 2002, Giménez et al. 2010). The mammary gland tumors account for 17% of all tumors diagnosed in cats (Lana 2007), with 85% of lesions categorized as malignant, exhibiting high invasiveness and causing a high mortality rate (Mills et al. 2015). Furthermore, malignant mammary gland neoplasia ranks as the third most frequent feline neoplasia, following lymphoma and skin tumors (Dorn et al. 1968, Lana 2007).

In queens neutered between the ages of 6-12 months, the risk of neoplasia is estimated to be between 9-14% (Overley et al. 2005). In non-neutered queens, mammary gland neoplasia most frequently occurs in cats over 12 years old (Zappulli et al. 2015), but it also occurrs in early neutered queens at the age of 12-16 (Giménez et al. 2010) suggesting that some factors, other than ovarian steroids, play a causative role in mammary tumorigenesis in female cats.

Among endogenous factors influencing the development of feline mammary gland neoplasia, prolactin (PRL) and vascular endothelial growth factor (VEGF) are noteworthy. PRL plays a role in physiological mammary gland development and the regulation of lactation. PRL is a single-chain peptide hormone that functions in the organism both as a blood-secreted hormone and as a cytokine. It is synthesized in the anterior pituitary as well as in other tissues such as the ovaries, endometrium, placenta, mammary gland, lymphocytes, and neoplastic cells (Marano 2014, Skałba 2016). PRL acts on the target organs via its specific receptor – prolactin receptor (PRL-R), which belongs to the superfamily of first-class cytokine receptors (Bole-Feysot et al. 1998). As a cytokine, it functions as an immunomodulating particle, triggers growth and differentiation of the cells, and inhibits apoptosis (Badowska-Kozakiewicz 2012).

PRL plays a significant role in various locations of neoplasia including the mammary gland, prostate, colon, and rectum due to its local secretion and accumulation in the tissues (Leav et al. 1999) as it activates oncogenes, particularly Ras oncogene (Levina et al. 2009). Experiments on laboratory animals showed that PRL might play a role in cancer cell proliferation (Hanahan and Weinberg 2011). Experiments on mice indicated that PRL might inhibit the expression of transforming growth factor beta (TGF ß), which works by matrix metalloproteinases inhibition, which in turn plays a role in metastasis (Philips and McFadden 2004). The significant role of PRL in metastasis has been confirmed in bitches, as higher PRL concentrations were measured in the cell homogenate in clinically malignant cases (Queiroga et al. 2014). It has been shown that PRL induces mammary gland cancer development, most likely by the expression of genes that disturb the specific function of its receptor (Fernandez et al. 2010). Supposedly, PRL-PRL-R pathway activation is important for the initiation and progression of neoplasia in mammary gland cancer but is not the main factor behind the process of neoplasia itself (Gill et al. 2001, Peirce et al. 2001). PRL stimulates angiogenesis by initiating and supporting certain stages of new blood vessel formation. This directly influences endothelial cells, VEGF activity and the FGF 2/STAT 5 cascade (Clapp et al. 2008). VEGF is crucial for the formation of blood and lymphatic vessels which is particularly important in the case of mammary gland tumors (Köllermann et al. 2001). Therefore, considering both PRL-R and VEGF expressions in various types of mammary tumors in queens will provide new knowledge about the role of PRL and angiogenesis in the pathogenesis of feline mammary gland neoplasia.

This study aims to determine the expression of PRL-R and VEGF in relation to the histological type of the tumor in the mammary gland of female cats. The values of PRL-R and VEGF expressions were compared to identify relationships with clinical parameters, such as the intensity of tumor infiltration with inflammatory cells, the generalization of the neoplastic process, and ovariohysterectomy.

Materials and Methods

Animals

The study involved 60 queens of various breeds, with ages ranging from 4-18 years (mean age: 10.9 years) with a mammary gland tumor. The history was collected using a questionnaire, which included: age, breed, ovariohysterectomy date, parturition count, the date when the lesion was first noticed, and its growth rate. A complete clinical examination, basic blood morphology test, thoracic x-ray, abdominal and mammary gland ultrasound examination and echocardiography was conducted before the queens underwent an unilateral mastectomy. In cases of non-neutered animals, ovarohisterectomy was mandatory during the mastectomy procedure. Variables considered were generalization of the neoplastic process, including local recurrence and distant metastasis, and the time of

ovariohysterectomy. The entire resected neoplastic glands were used in histopathology and immunohistochemistry examinations. Sample collection was a part of standard veterinary diagnostic procedures in accordance with Polish legal regulations (Dz.U.2018.0.1207, art 1.2 (5)15.01.2015: Resolution on animal protection used for scientific and educational purposes). The approval of the Local Commission for Ethics in Animal Experiments was not required.

Histological staining and examination

The samples were fixed immediately after collection, using 4% formaldehyde for 24 hours, and then saturated in 70% ethyl alcohol. Paraffin blocks were cut into 6μm sections, fixed on silanized microscope slides, stained with hematoxylin and eosin (HE) and mounted with Canadian balsam resin.

HE stained slides were examined using a BX-61 (Olympus, Japan) light microscope with a LiveView archive system. Samples were histologically graded according to the WHO guidelines (Meuten 2017), based on the tumor type and malignancy into one of the following groups: group I – control, samples of mammary gland tissue showing no lesions or inflammatory changes; group II – non-neoplastic lesions – dysplastic and hyperplastic lesions to the mammary gland; group III – benign neoplastic tumors – simple and complex adenomas, fibroadenomas; group IV – malignant neoplastic tumors – adenocarcinomas of various malignancy (grade I - ^{o}I, grade II - ^{o}II, and grade III - ^{o}III). Inflammatory infiltration was described using a 4-grade scale including '-' as no infiltration, '+' as small infiltration, '++' as medium infiltration, and '+++' as intense infiltration.

Immunofluorescent labeling and evaluation

The corresponding slides were labeled using the protocol for immunofluorescence including incubation in citrate buffer (2 x 4 min, Avantor Performance Materials, Poland), and incubation in 1% bovine serum solution (1 x 1h, Sigma-Aldrich, Poland). Two monoclonal antibodies were used independently on two corresponding slides against PRL-R (dilution 1:300, 3h of incubation, goat anti-PRL-R antibody, E-20, Santa Cruz Biotechnology, USA) and VEGF C1 (dilution 1:300, 3h of incubation, mouse anti-PRL-R antibody, C-1, Santa Cruz Biotechnology, USA) antigens, respectively. The immunological reaction was visualized using secondary fluorochrome-labeled antibodies (dilution 1:500, 1 h, AlexaFluor 568, Life Technologies, Poland) anti-goat and anti-mouse, respectively. Finally, nuclei were stained with HOECHST 33342 (20 μg/ml, 2 min, Sigma-Aldrich, Poland). Omission of primary antibodies in negative control was used in order to confirm the specificity of antibody binding. Slides were qualitatively examined using a Leica SP8-WLL confocal microscope (KawaSka, Poland) and quantitatively analyzed using a ScanarR (Olympus, Japan) scanning cytometer. Slides were observed under the confocal microscope using 20x and 63x magnification objectives, digital zoom, excitation wavelength 405 nm for Hoechst 33342 and 578 nm for AlexaFluor 568, and emission wavelength 420-450 nm for Hoechst 33342 and 580-630 nm for AlexaFluor 568. The reaction was considered positive when red fluorescence around the nuclei was found. Immunofluorescent reactions were quantized in a scanning cytometer using a 20x magnification objective, triple band excitation and emission filter (DAPI/FITC/TxR). Nuclei were detected in the DAPI spectrum and AlexaFluor 568 fluorescence was detected in the TxRed spectrum. The reaction was considered positive when a mean fluorescence intensity (MFI) above the background threshold value was noted in the nuclei and at a distance of 10 μm around nuclei. The minimal number of cells counted from each sample was 20000 in a minimum of 1000 scans. The MFI threshold value was established based on the negative control.

Data analysis

Data distribution was tested using the Shapiro-Wilk. Two data sets were compared using the unpaired t-test with Welch's correction or the Mann-Whitney test. More than two data sets were compared using one-way ANOVA or the Kruskal-Wallis test with Tukey's multiple comparison test or Dunn's multiple comparison test, respectively. In the case of the expression PRL-R and VEGF in different types of mammary gland tumors, the two-steps quick cluster analysis was performed to the established threshold and mean values for low, moderate, and high PRL-R and VEGF expression. Correlations were calculated with the Pearson correlation coefficient (ρ) or the Spearman correlation coefficient (Sr). Results were considered significant for $p<0.05$. Results were reported on plots as mean ± SEM. Statistical analyses were performed in GraphPad Prism 6 software (GraphPad Software Inc., USA).

Results

Clinical data and histopathology diagnosis

The mean diameter of the mammary gland tumors ranged from 0.4 to 6.0 cm. The presence and location of smaller lesions were confirmed with ultrasonography. In 33 samples (55% of cases) malignant adenocarcino-

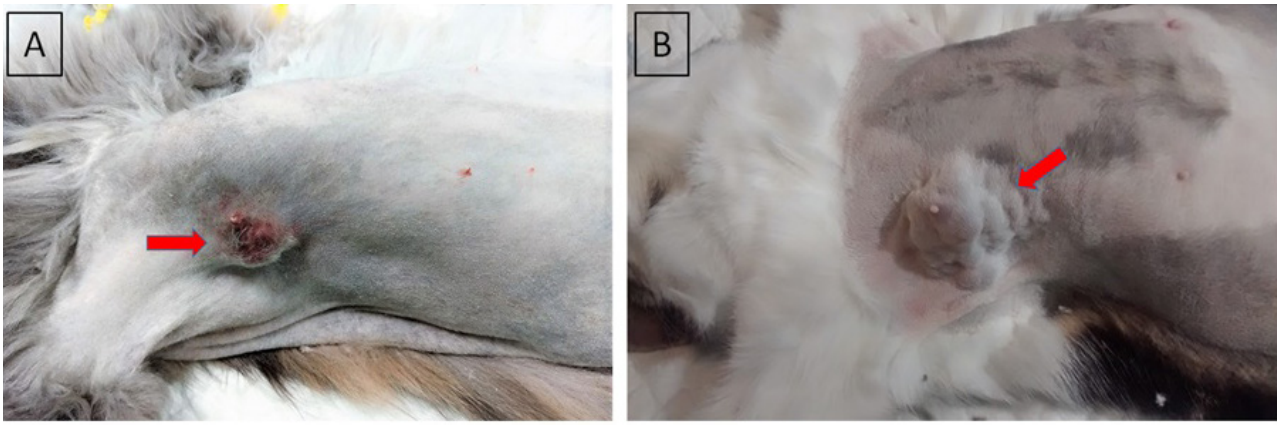

Fig. 1. Macroscopic images of mammary gland tumors in the female cats. A tumor (red arrow) of the first mammary gland. The superficial ulceration is present. In histopathological examination, grade III simple adenocarcinoma was diagnosed. A female cat at the age of 9 (**A**); A tumor (red arrow) of the first mammary gland. Visible cystic changes in tumor. In histopathological examination, dysplastic changes including cystic hyperplasia. A female cat at the age of 13 (**B**).

Table 1. Threshold values (min, max) and mean values for low, moderate and high expression of prolactin receptor (PRL-R) and vascular endothelial growth factor (VEGF).

	Low expression (%)			Moderate expression (%)			High expression (%)		
Antigen	Min.	Max	Mean	Min	Max	Mean	Min	Max	Mean
PRL-R	0.04	14.71	5.92±4.38	17.04	33.48	26.04±5.55	41.75	60.37	49.68±7.75
VEGF	2.90	14.79	10.37±3.70	15.98	26.17	21.11±3.18	27.42	33.50	30.12±1.94

ma was diagnosed (group IV) (Fig. 1a). Simple adenocarcinoma was dominant (26 cases, 43% of total), including 21 cases of grade III malignancy, 3 of grade II malignancy, and 2 of grade I malignancy. The remaining adenocarcinomas were qualified as a solid carcinoma (1 sample), a cribriform carcinoma (3 samples), a carcinosarcoma (2 samples), and a comedocarcinoma (1 sample). In 16 tumors necrosis was observed, and in 4 tumors cysts were found. Ten adenomas (16%) were found, including 4 simple adenomas, 3 complex adenomas, and 3 fibroadenomas (group III). Dysplasia and hyperplasia (group II) were found in 12 cases (20%); however, only in 7 tumors, dysplasia was related to the neoplastic process. Among the dysplastic changes, tubular ectasia, lobular hypertrophy, tubular hyperplasia, cysts, and localized fibrosis were found (Fig. 1b). The remaining five samples were qualified to group I (serving as a control group). Twelve queens that developed malignant tumors were neutered on the day of mastectomy.

Expression of PRL-R and VEGF in different types of mammary gland tumors

The expression of PRL-R (Fig. 2) and VEGF (Fig. 3) was confirmed in all samples regardless of the tumor type. The high expression of PRL-R was observed in the stroma neighbouring proliferating ducts and tubules. Low expression of PRL-R was found in the stroma of adenocarcinomas grade III. However, for the entire examined sample no significant differences (p=0.581) were found in PRL-R expression between normal mammary glands, non-neoplastic lesions, benign tumors, and malignant tumors (Fig. 4a). The expression of VEGF was observed mostly in the epithelium, while it was rarely visible in the stroma. There were also no significant differences (p=0.348) found in VEGF expression between different types of mammary gland tumors (Fig. 4b). Therefore, independent variables cluster analysis, including determination of threshold values and mean values for low, moderate, and high expression of PRL-R and VEGF (Table 1) was conducted. A gradual increase in the share of malignant lesions in the subsequent clusters representing low, moderate, and high PRL-expression, was found. 39.1% of adenocarcinomas, 21.7% of normal glands, 21.7% of dysplastic tissues, and 17.4% of adenomas were classified as a cluster of low PRL-R expression. 62.6% of normal tissue, 43.8% of adenocarcinomas, 12.5% of adenomas, and 6.3% of dysplasias were in a moderate PRL-R expression cluster. 50% of malignant tumors, 20% of benign lesions (neoplastic and dysplastic), and 10% of normal mammary gland tissue were classified into the high PRL-R expression cluster. Similarly, a gradual increase in the share of malignant tumors was found in the subsequent cluster groups, representing low, moderate, and high VEGF expression. 33.3% of normal mammary gland samples, 27.8% of malignant and benign tumors, and 11.1% of dysplastic lesions were classified as a low VEGF expression cluster. 50%

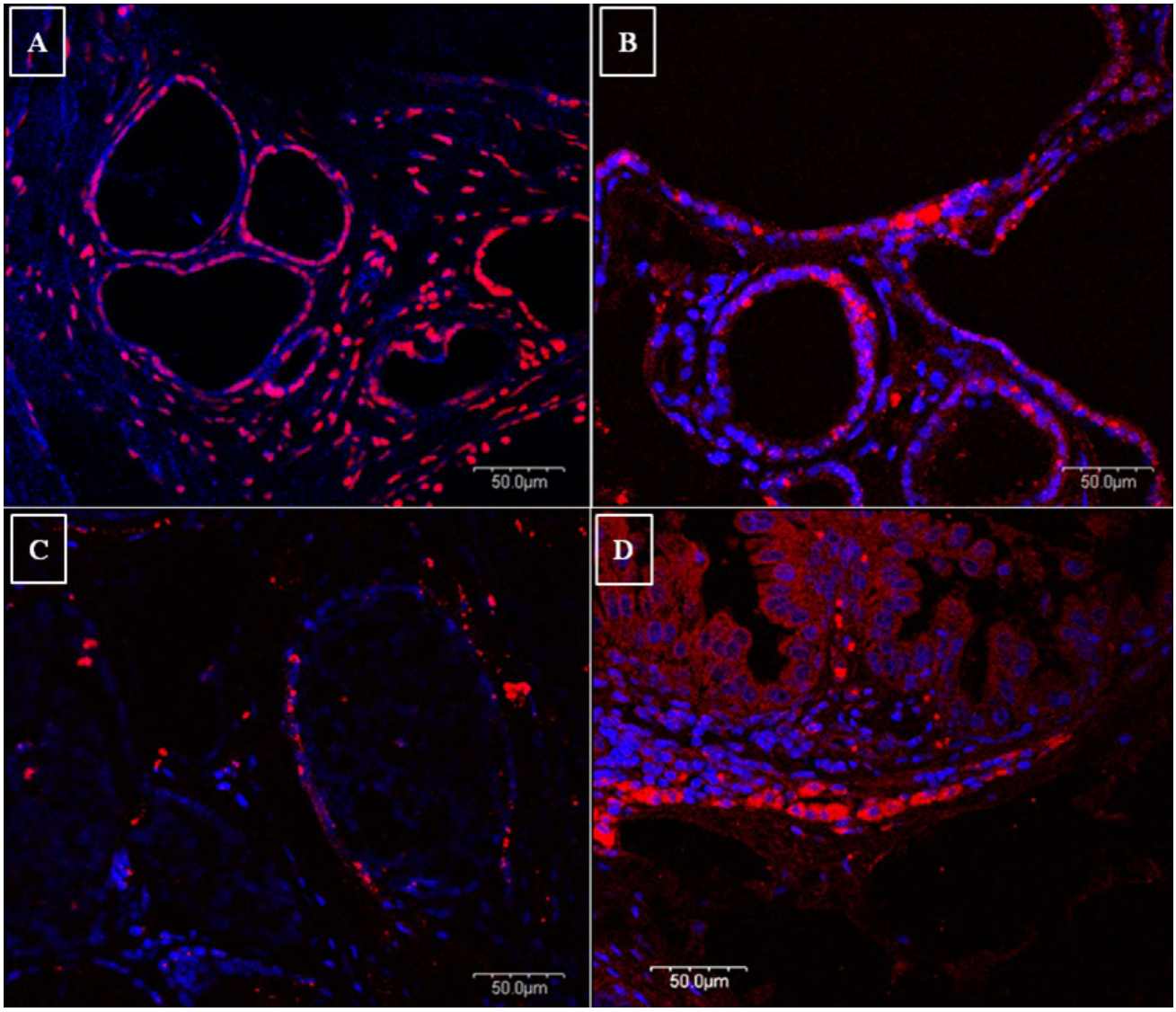

Fig. 2. The representative images of immunofluorescent labelling of prolactin receptor (PRL-R). Numerous immunopositive cells visible in normal mammary gland (control) (**A**); non-neoplastic lesions (**B**); benign neoplastic tumor (**C**); malignant.

of adenocarcinomas, 21.4% of normal tissue samples, 17.9% of dysplasias, and 10.7% of adenomas were classified as a moderate VEGF cluster. 63.6% of adenocarcinomas, 18.2% of adenomas, and 9.1% of normal and dysplastic mammary tissue samples were in the high VEGF expression cluster. Clusters of expression of PRL-R and VEGF and the share of each malignancy group are shown in Fig. 4 C-H.

No significant correlations between the expression of PRL-R and VEGF were found in the control group. Correlations for all examined tumor types are shown in Table 2.

Expression of PRL-R and VEGF and inflammatory infiltration intensity

Inflammatory infiltration showed predominantly lymphocytes, neutrophils, and macrophages. In the normal mammary gland either no infiltration, small infiltration, or medium infiltration was observed (Fig. 5A,E). In the normal mammary gland, only the expression of VEGF was lower ($p=0.043$) when the inflammation was medium in comparison to no inflammation (Fig. 5E). In non-neoplastic tumors no infiltration or small infiltration was noted (Fig. 5B,F), with lower expression of VEGF ($p<0.001$) when the inflammation was small (Fig. 5F), and with no differences in PRL-R (Fig. 5B). In benign (Fig. 5C,G) and malignant (Fig. 5D,H) neoplastic tumors all four grades of inflammatory infiltration intensity were found. No differences were found in benign neoplastic tumors. In malignant neoplastic tumors, the expression of PRL-R was lower ($p=0.019$) in medium and intensive inflammation than with small or no inflammation (Fig. 5D) while VEGF was higher ($p=0.009$) when the inflammation was intensive in comparison to no inflammation (Fig. 5H).

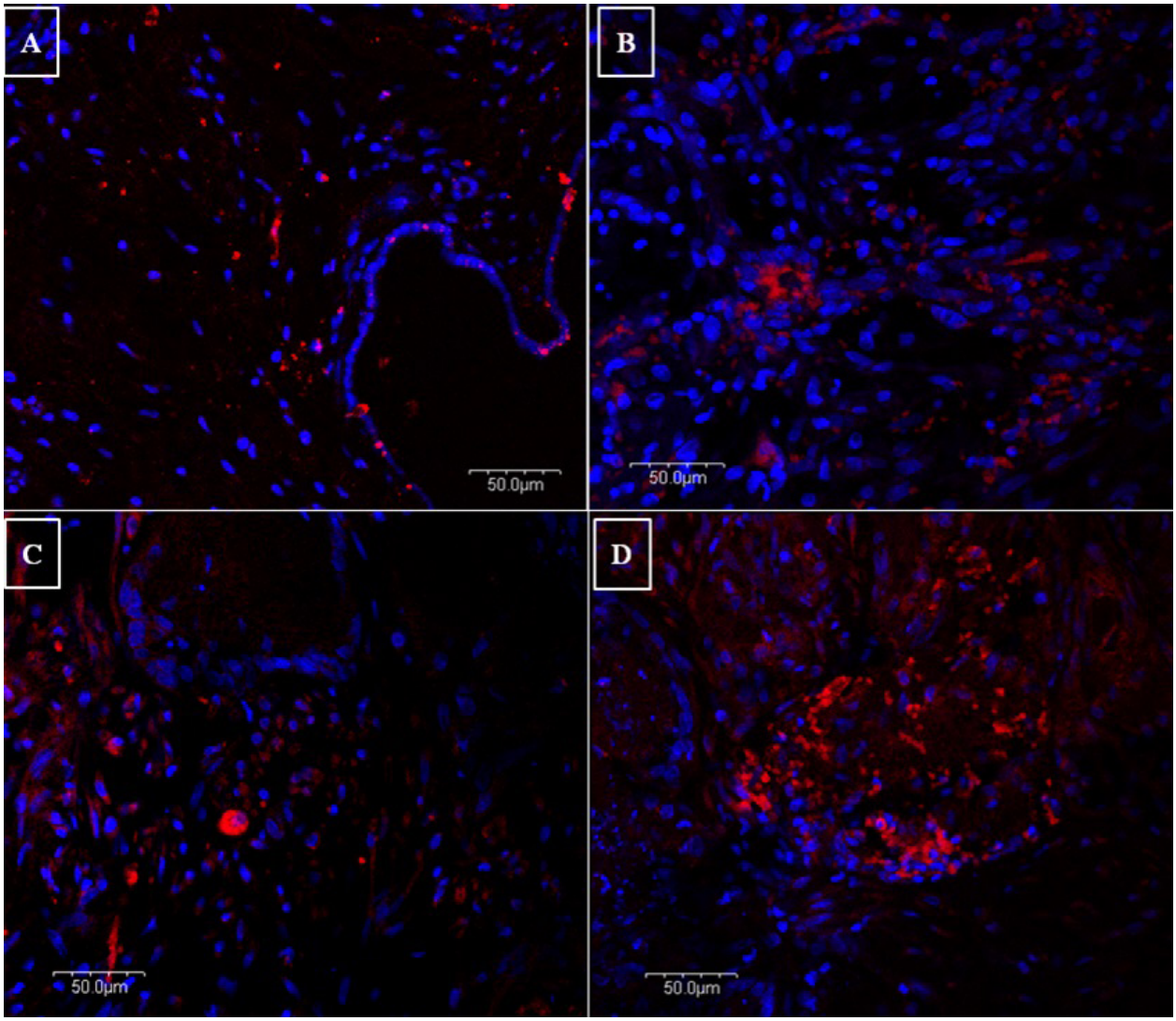

Fig. 3. The representative images of immunofluorescent labelling of vascular endothelial growth factor (VEGF). Numerous immunopositive cells visible in normal mammary gland (control) (**A**); non-neoplastic lesions (**B**); benign neoplastic tumor (**C**); and malignant neoplastic tumor (**D**). Emission spectra of AF 568/Hoechst. Confocal microscopy, magnification 20×.

Table 2. The Pearson correlation coefficient (ρ) and Range-Spearman correlation coefficient (Sr) between expression of prolactin receptor (PRL-R) and vascular endothelial growth factor (VEGF) in different types of mammary gland tumors. Correlations were considered statistically significant at $p<0.05$.

	Group I	Group II	Group III	Group IV
Coefficients	ρ = 0.15	Sr = -0.62	ρ = -0.77	Sr = -0.71
Probability value	p = 0.641	p = 0.015	p = 0.041	p = 0.008

Expression of PRL-R and VEGF and generalization of the neoplastic process

Local recurrence (Fig. 6A) and local or distant metastasis (Fig. 6B) were noted only in malignant neoplastic tumors. The expression of PRL-R (p=0.020) and VEGF (p=0.015) was higher with the distant metastasis than with lack of generalization and local recurrence (Fig. 7A,B).

3.5. Expression of PRL-R and VEGF and time of ovariohysterectomy

In non-neoplastic lesions and benign neoplastic tumors, no differences (p>0.05) in expression of PRL-R and VEGF were noted depending on the time of ovariohysterectomy. In malignant neoplastic tumor expression of PRL-R (p=0.018) was higher when queens were neutered before mastectomy. (Fig. 8).

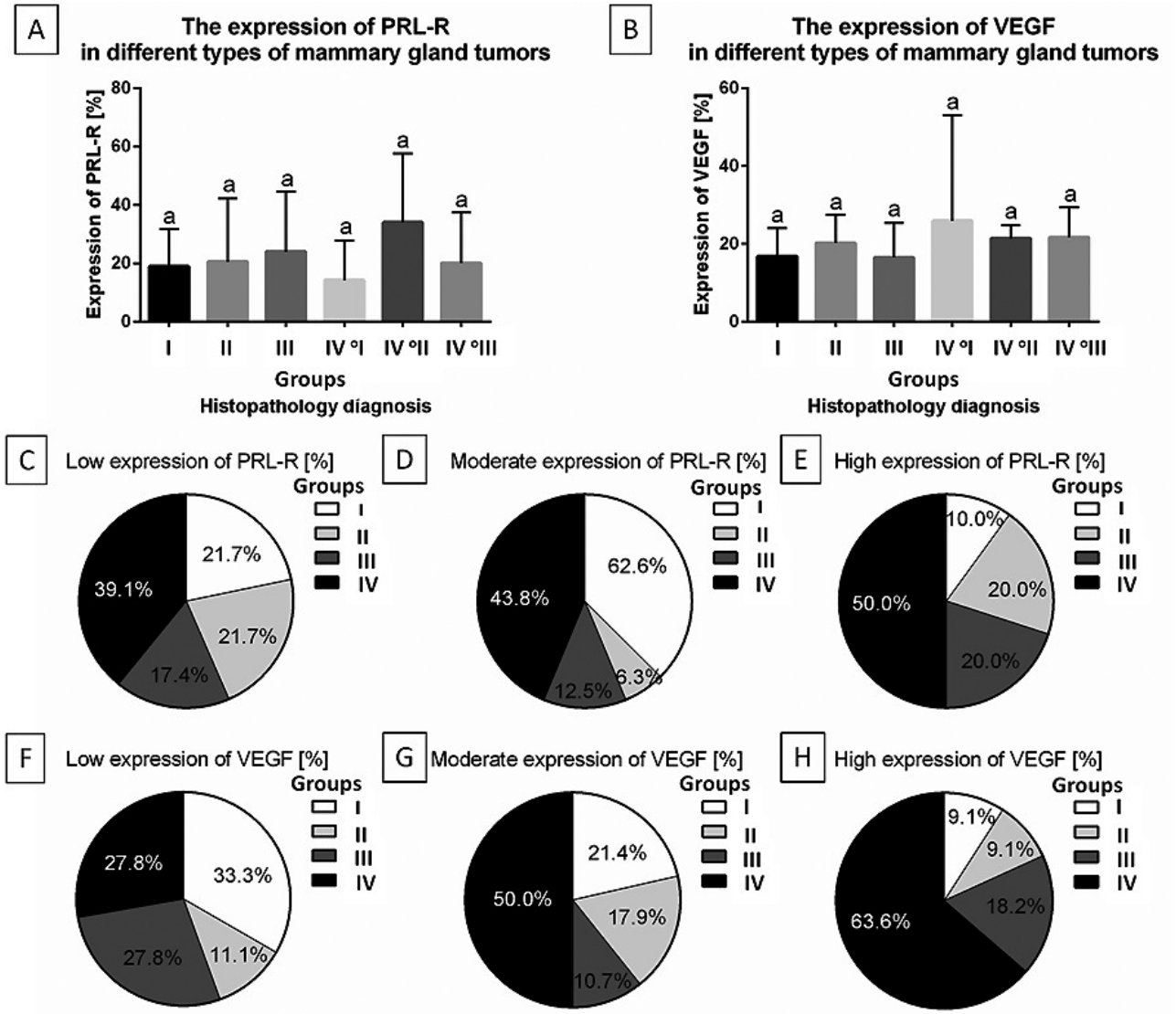

Fig. 4. The expression (mean +SD) of prolactin receptor (PRL-R) (**A**) and vascular endothelial growth factor (VEGF) (**B**) in different types of mammary gland tumors: group I, normal mammary gland (control); group II, non-neoplastic lesions; group III, benign neoplastic tumor; group IV, malignant neoplastic tumor (adenocarcinomas grades: °I, °II, and °III). Lower case letters indicate no differences between groups ($p > 0.05$). Independent variables clusters analysis of PRL-R (**C-E**) and VEGF (**F-H**) data series with the percentage of different types of mammary gland tumors in consecutive low expression (**C,F**), moderate expression (**D,G**), and high expression (**E,H**) clusters.

Discussion

Expression of PRL-R in different types of mammary gland tumors

Despite the lack of differences in expression of PRL-R among different types of mammary gland tumors, the results seem to confirm the cytokinetic function of PRL in queens, even though the peripheral blood or homogenated tissue concentrations were not evaluated. The lack of differences in the general expression of PRL-R among different tumor types can be explained by the high variability of the value expression and the lack of normal distribution of the studied subjects. Interestingly, in the applied expression cluster groups, as the expression of PRL-R grew, a gradual increase in the fibroadenoma share was observed, with up to 50% in the high expression cluster. In human malignant lesions, high PRL-R concentration was noted (Reynolds et al. 1997, Ferreira et al. 2008), while the opposite tendency was shown in dogs (Michel et al. 2012, 2014, Spoerri et al. 2015). Half of the malignant tumors (group IV) exhibit a high expression of PRL-R, which may imply an important role of PRL in carcinogenesis and prolactin-dependent modulation of the receptor expression in tissue. In the latest research conducted in humans, decreased transcription of the PRL-R in the mammary gland in response to high PRL concentration (both of pituitary and tissue origin) was shown

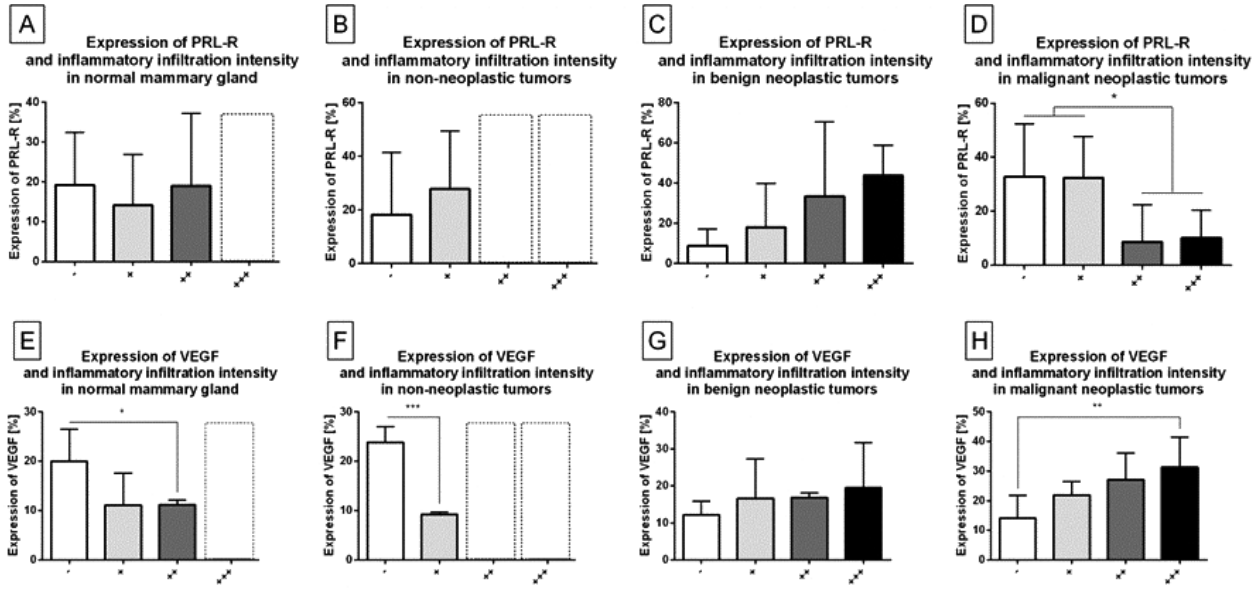

Fig. 5. Comparison between the expression of prolactin receptor (PRL-R) (**A-D**) and vascular endothelial growth factor (VEGF) (**E-H**), and the inflammatory infiltration intensity in different types of mammary gland tumors: group I, normal mammary gland (control) (**A,E**); group II, non-neoplastic lesions (**B,F**); group III, benign neoplastic tumor (**C,G**); group IV, malignant neoplastic tumor (**D,H**). Inflammatory infiltration was described using 4-grade scale including "-" as no infiltration, "+" as small infiltration, "++" as medium infiltration, "+++" intense infiltration. Stars indicate differences between groups of consecutive inflammatory intensity (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

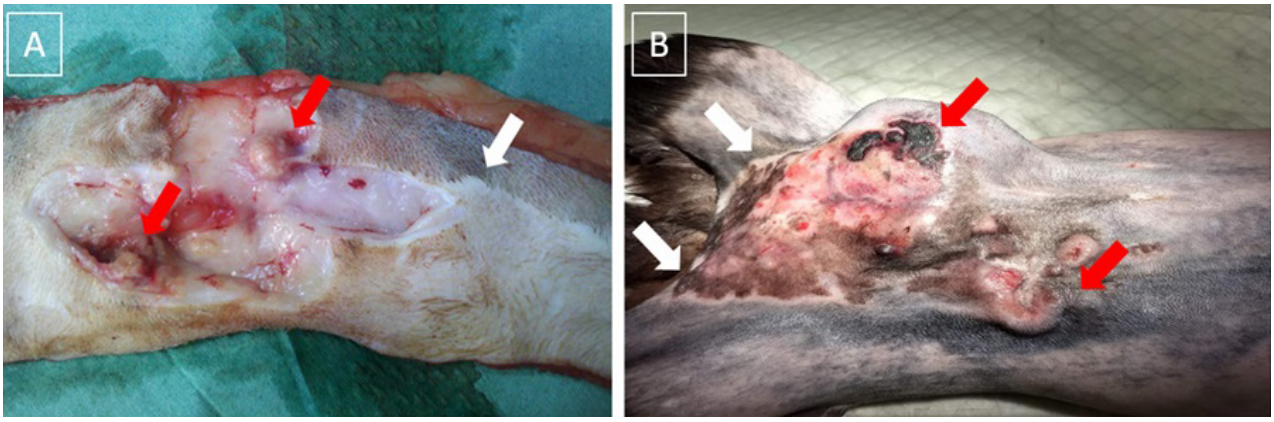

Fig. 6. Macroscopic images of malignant mammary gland tumors in the female cats. Generalization of the neoplastic process in the form of local recurrence (red arrows) in the postoperative scar (white arrow) (**A**); and the local metastasis in the form of massive tumor infiltration into the skin (red arrows) and into the inguinal lymph node (white arrows) (**B**). In histopathological examination, grade II adenocarcinoma was diagnosed . A female cat at the age of 11 (**A**). In histopathological examination, grade III adenocarcinoma was diagnosed. A female cat at the age of 14 (**B**).

(Li et al. 2006, Skałba et al. 2016). These results allow the assumption that neoplastic mammary gland cells show loss of the ability to differentiate and decrease of response to the para- /autocrine function of PRL. There are multiple examples of studies confirming the aforementioned phenomenon in women, bitches, and queens (van Garderen et al. 1999, Nieto et al. 2000, Giziński et al. 2004, Meuten 2017). Defective PRL-R response to PRL leads not only to its accumulation in the tumor tissue but also elongates and increases their response to PRL. This response in turn is involved in the transformation of glandular epithelium cells in the mammary gland in humans (Li et al. 2006, Plotnikov et al. 2009, Harvey et al. 2015), which may also take place in queens. In malignant mammary gland tumors in bitches, decreased expression of the PRL-R coding gene and increased peripheral blood and tissue homogenate PRL concentration have been shown (Sorenmo 2003, Klopfleisch et al. 2011, Spoerri et al. 2015).

Expression of VEGF in different types of mammary gland tumors

According to the literature containing similar evaluations, 90.5% of adenocarcinomas are VEGF-

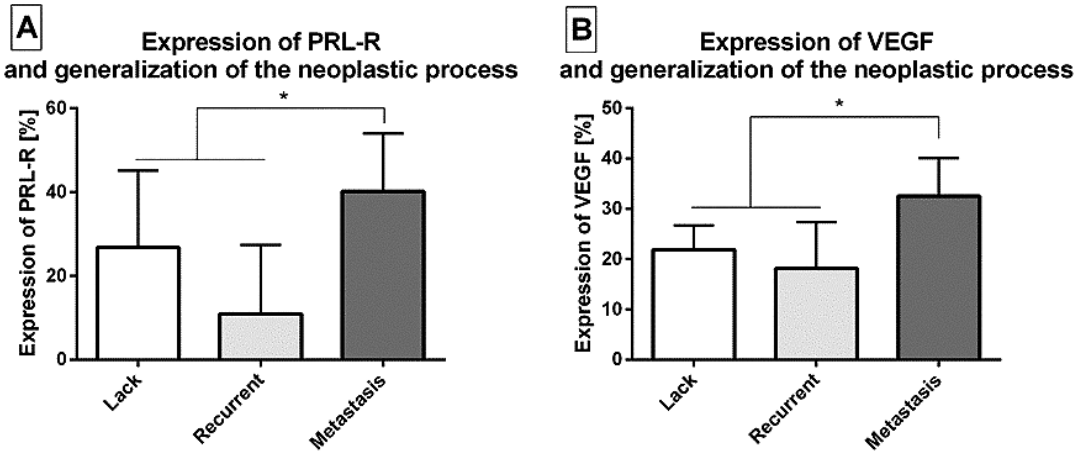

Fig. 7. Comparison between the expression of prolactin receptor (PRL-R) (**A**) and vascular endothelial growth factor (VEGF) (**B**), and generalization of the neoplastic process in malignant neoplastic tumors. Generalization was described as a local recurrence (recurrent) and a distant metastasis (metastasis). Stars indicate differences between groups of consecutive generalization findings (* $p<0.05$).

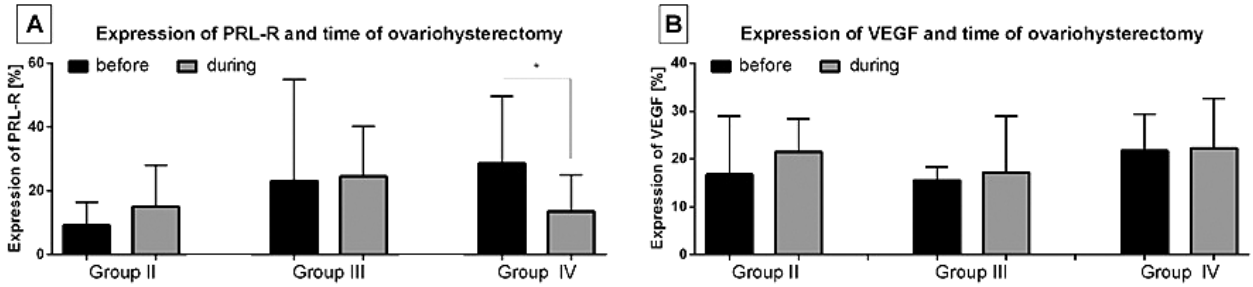

Fig. 8. Comparison between the expression of prolactin receptor (PRL-R) (**A**) and vascular endothelial growth factor (VEGF) (**B**) and time of ovariohysterectomy in different types of mammary gland tumors: group II, non-neoplastic lesions; group III, benign neoplastic tumor; group IV, malignant neoplastic tumor. The time of ovariohysterectomy was described as before investigated mastectomy – before, and during investigated mastectomy – during. Stars indicate differences between groups of consecutive ovariohysterectomy time (* $p<0.05$).

-positive, whereas the dysplastic lesions showed very low VEGF activity (Millanta et al. 2002, Islam et al. 2012). The highest quantity of VEGF-positive cells in previous findings was noted in the first-grade adenocarcinoma cluster (group IV, °I) and the lowest in normal mammary tissue (Karten 1998, Islam et al. 2012). Nonetheless, in this study differences among tumor-type clusters were not found. This lack of differences might be caused by small group size and a high variability of the age and breed of the specimens and the stage of the disease. The applied distribution analysis, which involves the cell VEGF expression share in the assessed tumors, indicated an increase in the expression in malignant tumors (group IV). The expression of VEGF in tumors was found mostly in the stromal epithelium, but also in the stroma itself, though less pronounced. This confirms the hypothesis that the growth and development of tumors are dependent on the development of the blood vessel system, in direct proportion to its proliferative activity (Griffoioen et al. 2000).

Expression of PRL-R and VEGF in different types of mammary gland tumors

In the normal mammary gland samples (group I), no significant findings were observed concerning expression, and there were no notable correlations between PRL-R and VEGF. However, a negative correlation was found between glandular dysplasia (group II) and VEGF and PRL-R expression. This suggests that the higher expression of PRL-R in the tumors, the lower the expression of VEGF, and in turn the higher the expression of VEGF, the lower the ability of the tissue to respond to PRL. Negative, strong correlations were found between the expression of antigens in benign and malignant tumors (groups III and IV). The results imply that normal mammary gland tissue exhibits a restricted capacity to induce angiogenesis, conversely to dysplastic and neoplastic processes. As previously shown, the dysplastic changes are characterized by much denser vascularity, which increases as the differentiation occurs prior to the transformation of the hyperplasia to a malignant tumor (Jensen et al. 1982, Strum 1983). The function of prolactin in the

growth and involution of the normal mammary gland is crucial, both during the estrus cycle and lactation. Growth of the mammary gland occurs via induction of neovascularization and differentiation of the capillary network in the glandular epithelium and in the stroma of the normal gland. The impact of PRL on the mechanisms of neoplastic angiogenesis remains unclear, primarily owing to the intricate nature of the process. PRL is both a proangiogenic and an anti-angiogenic factor at once. Under the influence of proteases, PRL promotes the generation of vasoinhibins, which subsequently induces apoptosis. (Clapp et al. 2008). It is possible, that concomitant with the neoplastic transformation process in queens, the apoptosis may intensify, consequently weakening neoplastic neoangiogenesis (Carpini et al. 2010). To elucidate the role of PRL in the feline mammary gland, further investigation is necessary, encompassing not only the examination of its receptor expression but also the assessment of peripheral blood and glandular tissue concentrations.

Expression of PRL-R and VEGF and generalization of the neoplastic process

In this study, local recurrence and distant metastasis were found in 87.9% of all cases. Most of the cited sources regarding the clinical features of the disease show that depending on different factors that determine mean survival, time equals ca. 10-12 months, and the disease-free interval (DFI) ranges from 95 to 1131 days (Estrela-Lima et al. 2010, Kim et al. 2013). Such short survival forces clinicians to seek prognostic markers of metastasis. Comparison between the antigen expression and occurrence of metastases in adenocarcinomas may be helpful in prognosis estimation. High expression of PRL-R and VEGF was noted only in cases of distant metastasis. The results obtained confirm the suspicion that the expression of PRL-R and VEGF may be treated similarly due to the strong proangiogenic function of PRL in mammary gland tumors. Proangiogenic factors released by the cancer cells, including PRL and VEGF, induce the formation of new blood vessels in the tumor so that the mechanism is not only autobut also paracrine (Ginsburg et al. 1995). Many studies suggest that VEGF expression is in relation to angiogenesis in the primary neoplastic lesion in mammary gland tumors in women via selective binding to the tyrosinase kinase in the epithelial cells. Tyrosinase kinase inhibitors are used in antiangiogenic therapy in many tumors in humans and animals. In studies on mammary gland tumors in queens, a strong correlation was shown between VEGF expression and clinical features. It was also shown that the microvessel density (MVD), the angiogenesis intensity indicator, was not related to the clinical parameters, including survival time. Our findings of the synergistic correlations of the PRL-R and VEGF expression and metastasis bring up a strong argument in favor of further studies regarding these antigens in the aspect of the future therapy of the mammary gland tumor in queens using antiprolactin and angiogenesis inhibition treatment.

Expression of PRL-R and VEGF and time of ovariohysterectomy

In this study, a higher proportion of malignant tumors (group IV) was observed in queens that underwent ovariohysterectomy before the mastectomy, while the proportion of adenomas (group III) was higher than that of group II. Data suggest that queens spayed after the age of 2 years are at a higher risk for malignant tumors compared to non-spayed queens (Overley et al. 2005). The protective effect of gonadectomy appears to be significant only when performed at an early age. These findings align with clinical observations, indicating that the impact of gonadectomy on the occurrence of malignant lesions in queens is not as pronounced as in female dogs. Notably, the feline mammary gland exhibits lower estrogen receptor expression than that observed in female dogs and women (Misdorp et al. 1991). During the progesterone-dominant phase, uncontrolled growth of mammary gland tissue may be attributed to the influence of progestins on the growth hormone--dependent pathway, mediated by an increase in the expression of growth hormone receptors and stimulation of paracrine release (Rutteman et al. 1989). Adenocarcinomas resected after ovariohysterectomy displayed a higher expression of PRL-R compared to cases where resection and ovariohysterectomy occurred simultaneously. Such differences were not observed for VEGF or other tumor types. The literature does not provide a clear rationale for the elevated expression of PRL-R in previously spayed queens. Presumably, the increased expression of PRL-R in these cases may be linked to peripheral and local hyperprolactinemia in the mammary gland resulting from gonadectomy.

Conclusions

The levels of PRL-R and VEGF expression exhibited no significant variation among the different histological types of mammary gland tumors in female cats. Nevertheless, clusters with high expression were more commonly associated with malignant neoplastic tumors. Lower PRL-R expression was observed with medium and high inflammatory intensity compared to those with non- and low inflammatory levels in adenocarcinomas. This suggests a potential role of PRL

in immune response modulation within feline malignant neoplastic tumors. The high expression of both PRL-R and VEGF was exclusively found with distant metastasis. The high expression of PRL-R in adenocarcinomas removed after ovariohysterectomy highlights the necessity for further exploration regarding the impact of gonadectomy in the prevention of mammary gland tumors.

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