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*Original article*

# Changes in serum neopterin and C-reactive protein concentrations in female dogs with mammary gland tumours

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## Abstract

The aim of this study was to measure serum neopterin and C-reactive protein (CRP) concentrations in female dogs with mammary tumours and evaluate the association between the values of these indicators and some clinical characteristics of the tumour. Fifty three female dogs were used for this study, including 43 dogs with mammary gland tumours (10 benign and 33 malignant) and 10 healthy controls. The concentrations of neopterin and CRP were determined using the ELISA technique and commercial ELISA kits. The mean serum neopterin concentration in female dogs with mammary tumours was lower than in healthy dogs, but significant difference was not found. Similarly, there were no significant differences in neopterin concentrations in female dogs based on tumour size, tumour ulceration and metastasis. The mean CRP concentration was significantly higher ( $p < 0.05$ ) in dogs with malignant tumours compared to dogs with benign tumours and control. Furthermore, serum CRP concentration was significantly higher ( $p < 0.05$ ) in dogs with metastatic malignant tumours compared to dogs with non-metastatic mammary tumours. The CRP concentration was significantly lower ( $p < 0.05$ ) in dogs with tumours less than 3 cm compared to those with larger tumours, and significantly higher in dogs with ulcerated tumours compared to those without ulceration. Our findings suggest that the neoplastic process in the mammary gland does not cause significant changes in serum neopterin concentrations in dogs. Higher concentrations of serum CRP in dogs with advanced stages of malignant tumours may suggest that CRP could be a potential prognostic marker in canine malignant mammary tumours, but this hypothesis needs further study.

**Key words:** neopterin, C-reactive protein, mammary gland tumours, female dogs

## Introduction

Mammary tumours are very common in older female dogs and show many similarities to breast cancer in women (Kumaraguruparan et al. 2006, Sleenckx et al. 2011). Over half of them are malignant and may metastasize, shortening the life of these dogs (Sleenckx et al. 2011). Because of the high incidence and often fatal course there is a need to find indicators that will enhance understanding of their biological behavior and determine the prognosis.

There is strong evidence from human studies that tumour growth can be controlled by the immune system (Vesely et al. 2011, Jing and Shapiro 2014) and that inflammation plays a key role in all stages of tumourigenesis (Fernandes et al. 2015). Several studies in humans suggest that determination of systemic immune activation and inflammatory markers in various neoplasms, including breast cancer, may provide useful information on clinical behavior of the tumour and patient prognosis and treatment (Yildirim et al. 2008, Kristensen et al. 2012, Stoenescu et al. 2015, Wulaningsih et al. 2015). In contrast, little is known about the usefulness of these markers in dogs with mammary gland tumours.

Neopterin belongs to a group of unconjugated pterins derived from guanosine triphosphate by guanosine triphosphate cyclohydrolase I (Hamerlinck 1999). Neopterin is mainly synthesized by activated monocytes/macrophages following stimulation by interferon-gamma cytokine (IFN- $\gamma$ ), which is released by NK cells and T-lymphocytes (Hoffmann et al. 2003). It is believed that neopterin production reflects the activity of immune system cells and may provide information about the status of cell-mediated immunity (Murr et al. 2002). Moreover, neopterin is involved in the modulation of oxidative stress and oncogene activation as well as in tumour growth, differentiation, and progression (Murr et al. 1999b, Sucher et al. 2010). Numerous studies in humans have demonstrated elevated serum neopterin levels in patients with various neoplasms and a positive correlation has been found between high levels of neopterin and poor prognosis (Weiss et al. 1993, Murr et al. 1999a, Schroecksnadel et al. 2006, Yildirim et al. 2008). However, data on neopterin levels in female dogs with mammary gland tumours are scarce (Tascene et al. 2011, Szczubiał et al. 2014).

C-reactive protein (CRP) is a non-specific acute-phase protein that increases on acute infection, tissue trauma, chronic inflammatory disease, surgery and neoplastic diseases (Ceron et al. 2005). It is secreted primarily by hepatocytes in response to cytokine stimulation, for instance IL-1, IL-6 and TNF $\alpha$  (Mortensen 2001). An elevated level of circulating CRP has fre-

quently been associated with increased incidence and worse outcome in various types of human cancer, including breast cancer (Allin et al. 2011, Han et al. 2011, Sicking et al. 2014). CRP is a major acute phase protein in dogs and may be used as a nonspecific marker of inflammation (Ceron et al. 2005). Increased concentrations of CRP have been found in dogs with lymphoma and other hematologic malignancies (Tecles et al. 2005, Mischke et al. 2007). However, very few reports are available on serum CRP concentration in female dogs with mammary gland tumours (Planellas et al. 2009, Tecles et al. 2009).

The aim of this study was to measure serum neopterin and CRP concentrations in female dogs with mammary gland tumours and evaluate the association between the values of these indicators and some clinical characteristics of the tumour.

## Materials and Methods

The study was performed in accordance with animal protection regulations (Animal Experimentation Act, 15<sup>th</sup> January 2015).

### Animals and design of study

Fifty three intact purebred or mixed-breed female dogs were used for this study including 43 dogs with mammary gland tumours (age ranging from 6 to 13 years) and 10 healthy controls (aged 3-8 years) with no history of neoplastic disease. The animals were patients of the Department of Animal Reproduction, Faculty of Veterinary Medicine in Lublin. The animals with mammary tumours were selected from a group of female dogs undergoing surgery due to spontaneously occurring mammary gland tumours. The control group animals were undergoing sterilization at the owner's request. None of the female dogs had any infectious diseases and had not used any drugs within 30 days prior to the surgical procedure.

Before surgery, all animals were clinically examined thoroughly and routine haematological and biochemical blood determinations and urine determinations were performed. Additionally, in the dogs with mammary tumours, three-view thoracic radiographs and abdominal ultrasound examinations were performed. In these animals, no other diseases were detected except for the mammary tumour. The animals of the control group were clinically healthy.

The presence of distal metastasis (yes/no), tumour size (< 3 cm vs. 3 – 5 cm vs. 5 cm), and the presence of tumour ulceration (yes/no) were recorded during physical examination.

Surgical resection of mammary tumours was per-

Table 1. Mean serum concentrations of neopterin and CRP in healthy dogs and dogs with mammary tumours.

Groups	No. of animals	Neopterin (nmol/L)	CRP ( $\mu\text{g/mL}$ )
BMT	10	2.08 $\pm$ 0.69	4.67 $\pm$ 2.12 <sup>a</sup>
NM-MMT	26	2.02 $\pm$ 0.95	21.90 $\pm$ 13.58 <sup>ab</sup>
M-MMT	7	2.60 $\pm$ 1.08	296.84 $\pm$ 97.34 <sup>ab</sup>
Control	10	3.05 $\pm$ 2.11	3.9 $\pm$ 3.4 <sup>b</sup>

<sup>a, b</sup> – the same letters mean statistically significant differences at  $p < 0.05$ . BMT - female dogs with benign mammary tumours, NM-MMT - female dogs with non-metastatic malignant mammary tumours, M-MMT - female dogs with metastatic malignant mammary tumours

formed according to standard practice, aiming to remove the tumour with complete margins. The sections of removed mammary tumours were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin blocks and sliced into 4  $\mu\text{m}$  sections. The microscopic preparations, stained with haematoxylin and eosin, were evaluated histologically according to the WHO classification of tumours and mammary gland dysplasia (Misdorp et al. 1999).

All female dogs with mammary tumours qualified for the study were diagnosed with a primary, single tumour. Based on clinical and histological findings, the animals were divided into three groups: (1) female dogs with benign mammary tumours (BMT,  $n=10$ ), (2) female dogs with non-metastatic malignant mammary tumours (NM-MMT,  $n=27$ ), and (3) female dogs with metastatic malignant mammary tumours (M-MMT,  $n=6$ ).

Nine millilitres of blood from all female dogs were collected from the cephalic vein immediately before surgery. The serum obtained after centrifugation was immediately frozen to  $-76^\circ\text{C}$  and kept deeply frozen until used for the determination of neopterin and CRP.

### Neopterin determination

The concentration of neopterin was determined using the ELISA technique and a commercial Neopterin ELISA kit (Immuno-Biological Laboratories, Hamburg, Germany) according to the manufacturer's instructions. The assay is reported by the manufacturer to have intra-assay CVs of 4.3 – 11.7% and inter-assay CVs of 8.8 – 13.8%. The absorbance was read using a microtiter plate reader (ELx800, BioTek Instruments, USA) at 450 nm.

### CRP determination

The CRP concentration was measured using a canine immunoassay (CRP ELISA, Tridelata Development Ltd, Kildare, Ireland) and a microtiter plate reader (ELx800, BioTek Instruments, USA) at 450 nm.

The assay is reported by the manufacturer to have intra-assay CVs of 6.9% and 6.5% and inter-assay CVs of 8.2% and 7.8%.

### Statistical analysis

Statistical analysis was performed using STATISTICA version 10.0 (Statsoft, USA). The one-way ANOVA test with the HSD Tukey's test or Student's  $t$ -test were used to determine significant differences in the concentrations of neopterin and CRP between the study groups. All values are expressed as the mean  $\pm$  standard deviation (SD). Differences at  $p < 0.05$  were considered statistically significant.

## Results

### Clinical and histological characteristics of mammary gland tumours

Among 43 mammary gland tumours, 10 were benign (6 complex adenomas and 4 benign mixed tumours) and 33 malignant (2 *in situ* carcinoma, 9 complex carcinomas, 8 tubulopapillary carcinomas, 4 solid carcinomas, 3 osteosarcoma, 1 fibrosarcoma, 6 carcinosarcoma). Distant metastasis (in lungs) were found in 7 dogs with malignant mammary tumours (1 solid carcinoma, 1 fibrosarcoma, 2 osteosarcoma, 3 carcinosarcoma). Among 33 malignant tumours, 6 were ulcerated (2 non-metastatic and 4 metastatic). The majority of malignant tumours were larger than 3 cm. Five tumours were less than 3 cm, 10 tumours had ranged from 3 cm to 5 cm, and 18 tumours were larger than 5 cm. All metastatic tumours and ulcerated tumours were larger than 5 cm.

### Serum neopterin concentration

The mean serum neopterin concentration in female dogs with mammary tumours was lower than in healthy dogs, but a significant difference was not found (Table 1). The concentration of neopterin in dogs with

Table 2. Mean serum concentrations of neopterin and CRP in dogs with different sized malignant mammary tumours (non-ulcerated and non-metastatic).

Groups	No. of animals	Neopterin (nmol/L)	CRP ( $\mu\text{g/mL}$ )
< 3 cm	5	1.80 $\pm$ 1.08	3.47 $\pm$ 0.72 <sup>ab</sup>
3 – 5 cm	10	2.30 $\pm$ 1.21	18.12 $\pm$ 8.48 <sup>a</sup>
> 5 cm	5	1.95 $\pm$ 0.90	39.65 $\pm$ 26.96 <sup>ab</sup>

<sup>a, b</sup> – the same letters mean statistically significant differences at  $p < 0.05$

Table 3. Mean serum concentrations of neopterin and CRP in dogs with ulcerated and non-ulcerated malignant mammary tumours.

Tumour	No. of animals	Neopterin (nmol/L)	CRP ( $\mu\text{g/mL}$ )
Ulcerated	6	2.06 $\pm$ 0.83	196.44 $\pm$ 72.24 <sup>a</sup>
Non-ulcerated	27	2.02 $\pm$ 0.89	17.92 $\pm$ 8.92 <sup>a</sup>

<sup>a</sup> – the same litters mean statistically significant differences at  $p < 0.05$

metastatic tumours was slightly higher compared to dogs with non-metastatic tumours and benign tumours, but significant differences were not found among these groups. In dogs with malignant tumours the mean serum neopterin concentrations did not differ significantly according to tumour size, although the value was higher in dogs with tumours larger than 3 cm (Table 2). When compared with neopterin concentrations in dogs with tumours of different sizes (excluding metastatic an ulcerated tumours), significant differences were not found. Similarly, there was no significant difference in neopterin concentrations in female dogs based on tumour ulceration (Table 3).

#### Serum CRP concentration

The mean CRP concentration was significantly higher ( $p < 0.05$ ) in dogs with malignant tumours compared to dogs with benign tumours and control (Table 1). Furthermore, serum CRP concentration was significantly higher ( $p < 0.05$ ) in dogs with metastatic malignant tumours compared to dogs with non-metastatic mammary tumours. No significant difference was observed between dogs with benign mammary tumours and control. The CRP concentration increased with the size of non-ulcerated malignant mammary tumours in dogs with non-metastatic disease. However, significant differences ( $p < 0.05$ ) were only found between dogs with tumours less than 3 cm and those with larger tumours (Table 2). The mean CRP concentration was significantly higher ( $p < 0.05$ ) in dogs with ulcerated tumours compared to those without ulceration (Table 3).

## Discussion

Anti-tumour immune response and inflammation in the microenvironment of the tumour are known to be important to the initiation and progression of neoplasms (Vesely et al. 2011, Fernandes et al. 2015). Therefore, examination of indicators of systemic immune activation and inflammation in humans and animals with malignant disease might provide information on tumour aggressiveness and clinical outcome (Sucher et al. 2010, Yildirim et al. 2008, Kristensen et al. 2012, Stoenescu et al. 2015, Wulaningsih et al. 2015). In this study we examined serum levels of neopterin and CRP in female dogs with benign and non-metastatic and metastatic malignant mammary tumours.

The results of our study show that the mean serum neopterin concentrations in dogs with mammary tumours did not differ significantly from that in the control group. Moreover, there was no significant differences between dogs with benign and malignant tumours, nor between dogs with metastatic and non-metastatic tumours. These findings seem to suggest that the neoplastic process in the mammary gland does not cause significant changes in serum neopterin concentrations in dogs. Our previous study showed significantly lower serum neopterin concentrations in dogs with non-metastatic carcinomas and sarcomas than in healthy controls (Szczubiał et al. 2014), while the study by Tascene et al. (2011) demonstrated similar serum neopterin concentrations in female dogs with malignant mammary tumours and healthy dogs.

The same clinical characteristics of canine mamma-

ry tumours, such as size and ulceration, have been associated with greater aggressiveness and poor prognosis (Yamagami et al. 1996, Matos et al. 2012). We found no significant differences in neopterin concentrations between dogs with ulcerated and non-ulcerated mammary tumours, and also none among dogs with different sized tumours. These findings indicate that serum neopterin level is not an indicator of clinical tumour aggressiveness in dogs with malignant mammary tumours.

The results of studies on serum neopterin levels in women with breast cancer are controversial. Some authors found increased serum neopterin levels in some breast cancer patients in comparison to patients with benign breast lesions and healthy controls (Murr et al. 1999a, Demian et al. 2014), but others reported that serum neopterin levels were similar in patients with primary breast cancer without metastasis, patients with benign breast lesion and controls (Yildirim et al. 2008).

Inflammatory response plays a key role in all stages of tumorigenesis, including initiation, promotion, growth, invasion, and metastasis, also affecting immune surveillance (Fernandes et al. 2015). Chronic inflammation may initiate and promote cancer through generation of reactive oxygen species and proinflammatory cytokines and, conversely, inflammation may occur secondary to cancer and affect disease progression (Asegaonkar et al. 2015, Fernandes et al. 2015). Inflammation enhances proliferation and survival of breast cancer cells by networks of cytokines and promotes tumour angiogenesis (Jing and Shaphiro 2014). Many studies in humans have explored the relationship between inflammation and various neoplasms, including breast cancer, through the use of systematic inflammatory markers, such as C-reactive protein (Allin et al. 2011, Sicking et al. 2014, Asegaonkar et al. 2014, 2015). In contrast, data on serum concentrations of CRP in dogs with mammary tumours are limited. Our study, as well as studies of other authors (Planellas et al. 2009, Tecles et al. 2009), showed no difference in serum CRP concentrations between the group of female dogs with benign mammary tumours and healthy dogs. These results suggest that benign canine mammary tumours are poor stimuli for production of CRP. However, we found that CRP concentration was significantly higher in dogs with malignant mammary tumours compared to those with benign tumours and healthy controls. Similarly, higher concentrations of CRP in dogs with mammary carcinomas and fibrosarcomas than in dogs with benign mammary tumours and healthy dogs were found by Planellas et al. (2009). In addition, the concentration of CRP was significantly higher in dogs with metastatic tumours, ulcerated tumours and

those with tumours larger than 3 cm. Our results are consistent with the results of a study conducted previously by Tecles et al. (2009). Because tumour size, ulceration and metastasis are recognized prognostic factors in dogs with malignant mammary tumours (Yamagami et al. 1996, Matos et al. 2012), it can be hypothesized that CRP could be a prognostic marker in canine malignant mammary tumours, but this hypothesis needs further studies.

Our results support the data from human medicine. In most studies CRP levels have been found to be higher in women with breast cancer than in those with benign diseases and healthy controls (Asegaonkar et al. 2014, Stoenescu et al. 2015, Wulaningsih et al. 2015). In many studies elevated CRP levels have been associated with larger tumour size, presence of distant metastases and poor prognosis (Han et al. 2011, Asegaomhar et al. 2014, Stoenescu et al. 2015, Wulaningsih et al. 2015).

## Conclusion

Our findings suggest that a neoplastic process in the mammary gland does not cause significant changes in serum neopterin concentrations in dogs. Higher concentrations of serum CRP in dogs with advanced stages of malignant tumors may suggest that CRP could be a potential prognostic marker in canine malignant mammary tumours, but this hypothesis needs further studies.

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