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Original article

Plasma interleukin-1 α and interleukin-8 in female dogs with non-metastatic and metastatic malignant mammary gland tumours

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Abstract

In this study plasma concentrations of IL-1 α and IL-8 in 29 female dogs with malignant mammary gland tumours (19 without metastasis and 10 with metastasis) and in 10 healthy control animals were determined. Concentrations of IL-1 α and IL-8 were analysed using a specific canine ELISA assay. Mean plasma concentrations of IL-1 α and IL-8 were significantly higher ($p < 0.05$) in female dogs with both non-metastatic and metastatic malignant tumours compared to the healthy animals. The concentrations of both tested cytokines were significantly increased ($p < 0.05$) in the dogs with metastasis. In female dogs with mammary carcinomas, the plasma concentration of IL-1 α was significantly higher ($p < 0.05$) in the animals with grade 3 tumours compared to the dogs with grade 1 tumours. The concentration of IL-8 was significantly higher ($p < 0.05$) in the dogs with grade 3 tumours compared to that found in the animals with grade 1 and grade 2 tumours. A moderate correlation ($r = 0.433$) was found between IL-1 α and IL-8 concentrations in the female dogs. These findings suggest that increased malignancy and invasiveness of canine mammary tumours is associated with an increased production of IL-1 α and IL-8 in the tumour microenvironment, which, in turn, leads to an increase in their circulating levels. This may indicate that circulating levels of the cytokines investigated could be considered as diagnostic and prognostic markers in canine malignant mammary tumours. However, further studies in this field are needed.

Key words: IL-1 α , IL-8, canine mammary tumours, plasma, metastases

Introduction

It is well known that tumour development and progression can be controlled by the immune system and that cytokines play an important role in this process (Fernandes et al. 2015, Irac et al. 2019). Various cytokines can both limit growth of the tumour by the activation of immune effector mechanisms and promote tumour development, and progression through their effect on apoptosis, angiogenesis, cell adhesion and transformation (Salvatore et al. 2017, Irac et al. 2019). It is believed that an increased production of various cytokines in the tumour microenvironment increases their circulating levels and might be utilized as a useful marker for the diagnosis and prognosis of tumours as well as for monitoring of the course of neoplasm (Andaluz et al. 2016, Li et al. 2017). The circulating levels of various cytokines, including IL-1 and IL-8, have been extensively studied in humans with breast cancer and other neoplastic diseases (Benoy et al. 2004, Chavey et al. 2007, Derin et al. 2007, Lyon et al. 2008, Li et al. 2017). In contrast, very few such studies have been performed in female dogs with mammary gland tumours.

Interleukin-1 (IL-1) represents a group of 17-20 kilodalton cytokines, the main representatives of which are proinflammatory cytokines IL-1 α and IL-1 β (Fasoulakis et al. 2018). IL-1 α is produced by many cell types, but endothelial and epithelial cells are the main source of this cytokine under both physiological and pathological conditions (Chiu et al. 2021). In tumour microenvironment IL-1 α can be produced by tumour cells themselves, infiltrating immune cells and tumour associated fibroblasts (Malik et al. 2018). The results of studies in humans suggested that IL-1 α exerts pro- and anti-tumour action in different cancers (Chiu et al. 2021). A significantly increased expression of IL-1 α has been found in some human cancers with distant metastasis as compared to those without metastasis (Baker et al. 2019). A circulating level of this cytokine has been significantly increased in the breast cancer patients (Lyon et al. 2008). The *in vitro* studies and the studies on animal models suggest that IL-1 α is involved in invasion and metastatic growth of breast cancer (Kumar et al. 2003, Holen et al. 2016, Kuan and Ziegler 2018).

Interleukin-8 (IL-8) is 8 kilodalton cytokine produced by macrophages and endothelial cells (Fasoulakis et al. 2018). IL-8 is a potent chemoattractant and activator of neutrophils and lymphocytes, and it is a key factor in tumour angiogenesis (Fasoulakis et al. 2018). IL-8 can be produced in the microenvironment of a tumour by tumour cells and by inflammatory cells (Raman et al. 2007, Fasoulakis et al. 2018). IL-8 expression in various human tumours, including breast

cancer, as well as serum concentration of IL-8 in patients with these tumours are significantly increased (Benoy et al. 2004, Chavey et al. 2007, Derin et al. 2007, Lyon et al. 2008, Li et al. 2017). The increased expression and higher serum levels of IL-8 indicate more invasive growth of breast cancer (Todorović-Raković and Milovanović 2013). Patients with a high serum IL-8 concentration had relatively lower survival rate than those with a low serum IL-8 concentration (Chen et al. 2012). It has been also shown that the addition of IL-8 to breast cancer cell lines promotes the invasion and chemotaxis of cancer stem cells (Ginestier et al. 2010).

Malignant tumours account for over half of mammary gland tumours, the most common neoplasms in intact female dogs (Sleeckx et al. 2011, Salas et al. 2015). They often are responsible for reducing dog lives and quality of life due to metastases to distant organs, mainly to the lungs (Sleeckx et al. 2011, Salas et al. 2015). It is accepted that canine mammary gland tumours share many similarities with breast cancer in women (Abdelmegeed and Mohammed 2018). Although, many studies have demonstrated that IL-1 and IL-8 play an important role in the development and progression of human breast cancer and their circulating levels are changed in breast cancer patients, the literature on serum/plasma concentrations of these cytokines in female dogs with mammary gland tumours is scarce. Thus, the purpose of this study was to assess the plasma levels of IL-1 α and IL-8 in female dogs with metastatic and non-metastatic malignant mammary gland tumours.

Materials and Methods

The study was performed in accordance with animal protection regulations (Animal Experimentation Act dated 15th January 2015).

Animals and design of the study

Thirty nine intact purebred or mixed-breed female dogs were used in this study including 29 dogs with malignant mammary gland (age ranging from 6 to 13 years) and 10 healthy animals, with no history of neoplastic disease, controls (aged 3-8 years). The dogs were patients of the Department of Animal Reproduction, Faculty of Veterinary Medicine in Lublin. The animals with malignant mammary gland tumours were selected from the group of female dogs provided by owners for treatment due to spontaneously occurring mammary gland tumours. The control group undergoing sterilization at the owner's request. All the owners gave informed consent to include their pets in the study.

We included female dogs with only one mammary gland lesion recognized as malignant tumour regardless of the tumour size or location, with no clinical signs of inflammatory mammary carcinoma and ulceration. To rule out other diseases, all the animals with mammary tumours were clinically examined thoroughly and routine haematological and biochemical blood determinations as well as urine determinations were performed. Moreover, in these dogs, three-view thoracic radiographs and abdominal ultrasound examinations were performed. The mammary tumours were examined carefully and measured. Regional lymph nodes were palpated. The aspirated samples for cytology examination using fine needle aspiration biopsy technique were performed in cases of lymphadenopathy.

In the dogs with non-metastatic tumours, the surgical resection of mammary tumours was performed according to standard practice, with an aim to remove the tumour with complete margins. Representative tissue pieces (from 3 to 6 depending on the size of the tumour) were collected from excised tumours and subjected to routine histological examination. In the dogs with metastatic tumours, the core needle biopsy was used to obtain tumour samples for histological examination. The diagnosis was confirmed by the examination of tumour samples collected after the animal had died or been euthanized.

The sections of mammary tumours were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin blocks and sliced into 4 μ m sections. The microscopic preparations, stained with haematoxylin and eosin, were evaluated histologically according to the Goldschmidt et al. (2011) classification. Malignant epithelial neoplasms were graded according to the Nottingham method for human breast tumours (Elston and Ellis 1998), adopted for canine mammary tumours (Pena et al. 2013).

The animals of the control group were clinically examined thoroughly and routine haematological and biochemical blood determinations, and urine determinations as well as abdominal ultrasound examinations were performed. All the animals in this group have had no history of mammary tumours or other neoplasms, and were clinically healthy. None of the dogs used for the study had use any drugs within 30 days prior to the sample collection.

Blood samples collection

Blood samples were taken as part of a routine healthy examination. Nine millilitres of blood from all the female dogs were collected from the cephalic vein into Vacuette tubes. The plasma obtained after centrifugation was immediately frozen to -76°C and kept deeply frozen until used for the determination of IL-1 α and IL-8.

Laboratory analysis

The plasma concentrations of IL-1 α and IL-8 were analysed using a specific canine ELISA assay (Cloud-Clone Corp., Houston, USA) according to the manufacturer's instructions. The absorbance was measured using a microtiter plate reader (ELx8000, BioTek Instruments, USA) at 450 nm. The detection limit of IL-1 α was less than 3.4 pg/mL. Intra- and inter-assay coefficients of variation were below 10 and 12%, respectively. The detection limit of IL-8 was less than 0.057 ng/mL. Intra-assay coefficients of variation was below 10% and inter-assay coefficients of variation was below 12%.

Statistical analysis

Statistical analysis was performed using the computer program STATISTICA version 10.0 (Statsoft, USA). The results were analysed for normal distribution using the Shapiro-Wilk test. The Kruskal-Wallis test followed by Mann-Whitney test with the Bonferroni correction was applied to determine significant differences in the concentrations of IL-1 α and IL-8 between the study groups. The one-way ANOVA test with the HSD Tukey's test were used to determine significant differences in the concentrations of these cytokines between the groups of dogs with various histological grade (grade 1-3) malignant epithelial mammary tumours. The correlations between IL-1 α and IL-8 concentrations in the dogs with malignant mammary tumours and the healthy dogs were presented using the Spearman's rank correlation coefficient. Differences at $p < 0.05$ were considered statistically significant.

Results

Clinical and histological characteristics of mammary gland tumours

The histopathological evaluation showed that in female dogs tubulopapillary carcinoma and complex carcinoma were the predominant types - 10 and 8, respectively. Five animals had solid carcinomas. Two tumours were diagnosed as *in situ* carcinomas, two as anaplastic carcinomas, and two as carcinosarcomas. Ten tumours metastasized (3 solid carcinomas, 4 tubulopapillary carcinomas, 2 anaplastic carcinomas, 2 carcinosarcomas). Seven tumors metastasized to the lungs and 3 tumours metastasized to regional lymph nodes and lungs. Among 27 malignant epithelial mammary gland tumours (carcinomas), 11 were grade 1 (G1) tumours, 11 grade 2 (G2) tumours and 5 grade 3 (G3) tumours. In the group of metastatic tumours histolo-

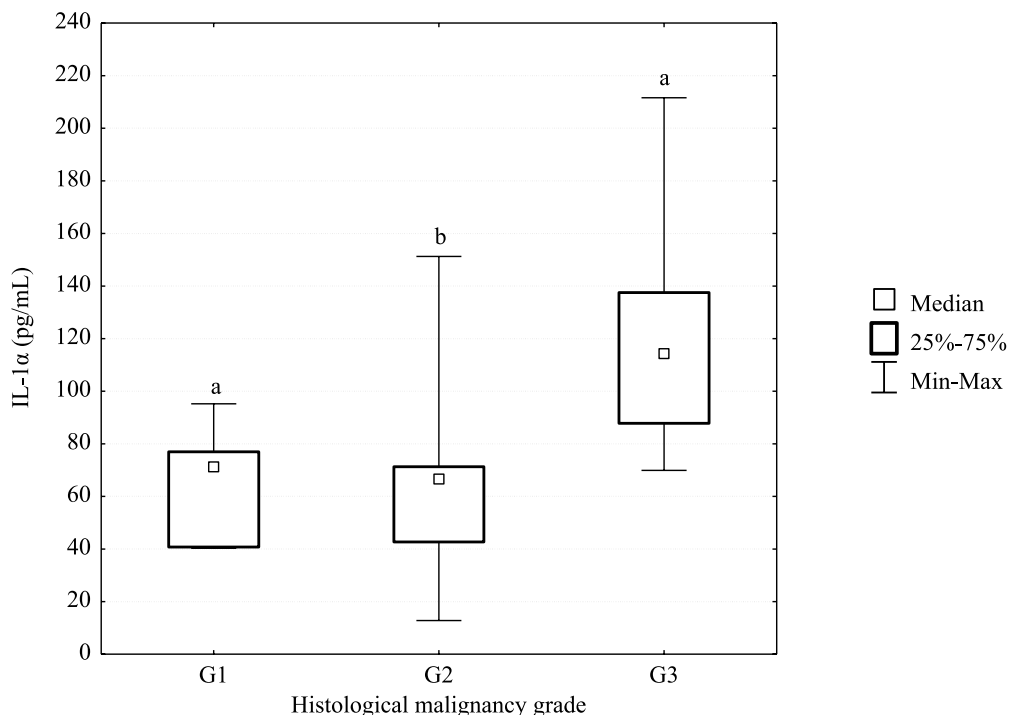


Fig.1. Box plot showing plasma concentrations of IL-1 α (pg/mL) in female dogs with various histological grade (G1-G3) malignant epithelial mammary gland tumours (carcinomas). ^{a,b} – the same litters mean statistically significant differences at $p < 0.05$.

Table 1. The mean plasma concentrations of IL-1 α and IL-8 in female dogs with malignant non-metastatic and metastatic mammary gland tumours, and in the healthy animals.

Group	Number of dogs	IL-1 α (pg/ml)	IL-8 (ng/ml)
Non-metastatic	29	52.10 \pm 44.92 ^a	0.55 \pm 0.42 ^b
Metastatic	10	105.37 \pm 46.92 ^a	1.42 \pm 0.98 ^b
Control	10	9.41 \pm 4.51 ^a	0.08 \pm 0.03 ^b

^{a,b} – the same litters mean statistically significant differences at $p < 0.05$.

gical grade 2 was found in 3 tumours and grade 3 in 5 tumours. Twelve tumours were 5 cm or less in size and 17 tumours had a size larger than 5 cm. All metastatic tumours had a size larger than 5 cm (from 6.5 cm to 13 cm).

Plasma IL-1 α concentration

The mean plasma concentration of IL-1 α was significantly higher ($p < 0.05$) in the female dogs with malignant tumours compared to the control group. In the dogs with metastatic tumours, the concentration of IL-1 α was significantly higher ($p < 0.05$) than in the dogs with non-metastatic tumours (Table 1). In the dogs with malignant epithelial tumours (carcinomas), the mean plasma concentration of IL-1 α was significantly higher ($p < 0.05$) in the animals with grade 3 tumours compared to the dogs with grade 1 tumours (Fig. 1). There were no significant differences in the concentra-

tion of IL-1 α between the dogs with grade 1 and grade 2 tumours, and between those with grade 2 and grade 3 tumours.

Plasma IL-8 concentration

Similarly as in the case of IL-1 α , the mean concentration of IL-8 was significantly higher ($p < 0.05$) in the dogs with malignant mammary tumours compared to the healthy dogs. The concentration of IL-8 was increased significantly ($p < 0.05$) in the dogs with tumours that metastasized compared to those with tumours that did not metastasize (Table 1). In the female dogs with grade 3 carcinomas, the concentration of IL-8 was significantly higher ($p < 0.05$) compared to the animals with grade 1 and grade 2 tumours (Fig. 2). There was no significant difference in IL-8 concentration between the grade 1 and grade 2 tumours.

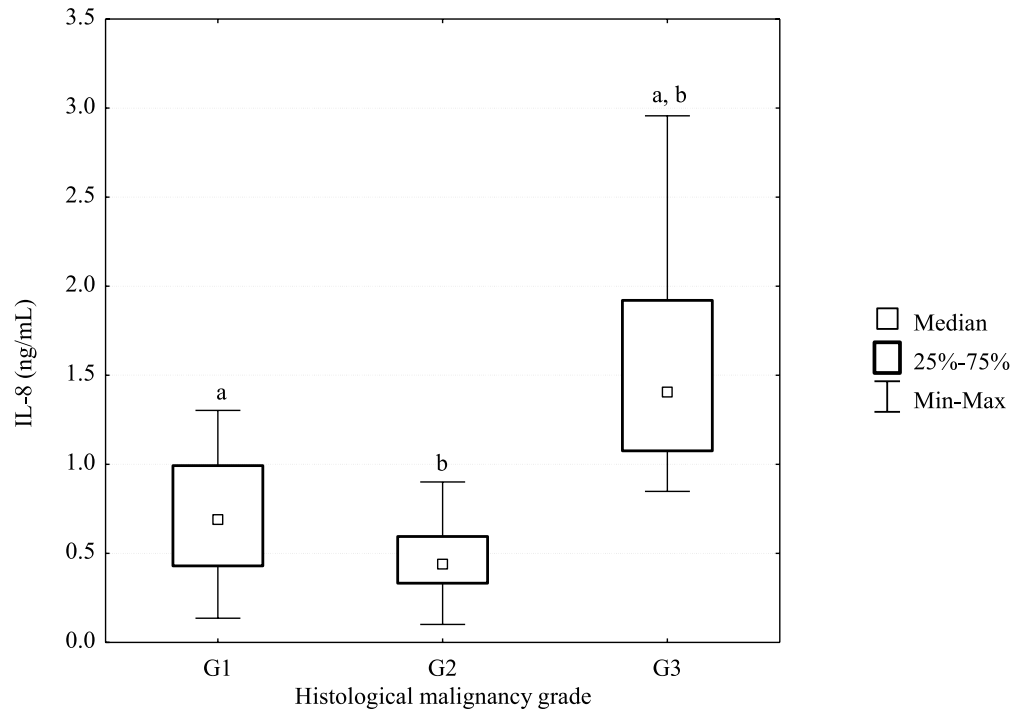


Fig. 2. Box plot showing plasma concentrations of IL-8 (ng/mL) in female dogs with various histological grade (G1-G3) malignant epithelial mammary gland tumours (carcinomas). ^{a,b} – the same litters mean statistically significant differences at $p < 0.05$.

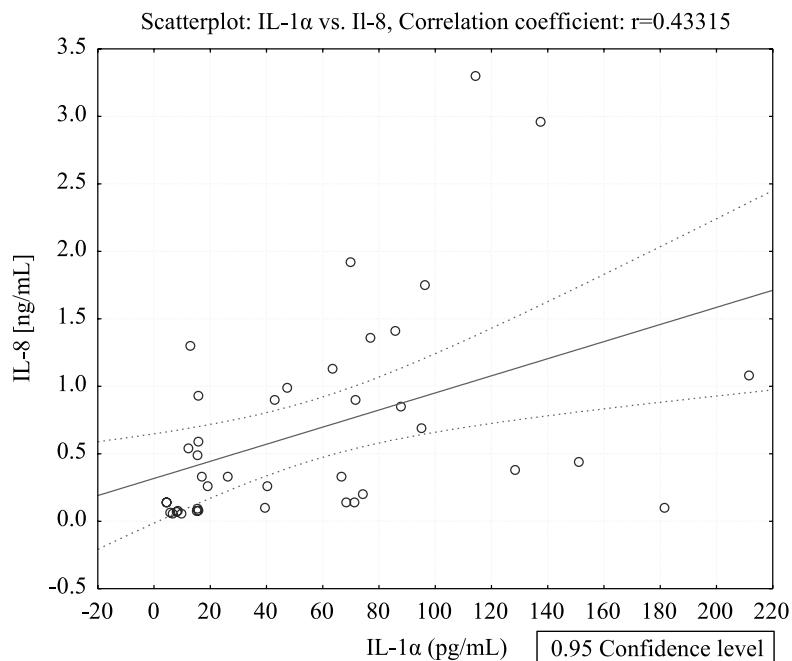


Fig. 3. Correlation between plasma concentrations of IL-1 α and IL-8 in female dogs with malignant mammary tumours and the healthy female animals.

Correlation between plasma IL-1 α and IL-8 concentrations

The correlation analysis showed a moderate correlation ($r=0.433$) between IL-1 α and IL-8 plasma concentrations in the female dogs (Fig. 3).

Discussion

In this study, the plasma IL- α and IL-8 concentrations in the female dogs with metastatic and non-metastatic malignant mammary tumours were determined. In addition, the relationship between concentrations of these cytokines and histological grade was ex-

mined. The results obtained indicate that the concentrations of both tested cytokines were higher in the dogs with malignant mammary tumours than in the healthy animals. Moreover, the concentrations of both cytokines were increased in the dogs with metastatic tumours compared to these with non-metastatic tumours.

To the authors knowledge so far only two studies have looked at the circulating level of IL-1 in female dogs with mammary tumours. According to the study by De Andres *et al.* (2013) the serum concentration of IL-1 α was increased in female dogs with mammary tumours compared to the healthy animals. The concentration of this cytokine was also significantly higher in the dogs with more malignant inflammatory mammary carcinomas compared to the animals with non-inflammatory mammary carcinomas. Similarly, Machado *et al.* (2015) have found a higher serum level of IL-1 in the dogs with mammary carcinoma compared to the healthy controls. The immunohistochemical study has demonstrated a higher expression of IL-1 in metastatic canine mammary carcinomas compared to non-metastatic mammary carcinomas and benign mammary tumours (Kim *et al.* 2010). These findings support the results of our study.

A few studies have shown higher circulating levels of IL-8 in female dogs with malignant mammary gland tumours compared to healthy animals and those with benign tumours (Gelaleti *et al.* 2012, De Andres *et al.* 2013, Estrela-Lima *et al.* 2013, Baba *et al.* 2019). According to the study by Gelaleti *et al.* (2012) a significantly higher IL-8 serum concentration is revealed in dogs with metastatic mammary tumours than the animals with non-metastatic mammary tumours. Similarly, a higher serum level of IL-8 has been found in women with metastatic breast cancer compared to the women with non-metastatic cancer (Benoy *et al.* 2004, Derin *et al.* 2007). In contrast, Monkong *et al.* (2020) have not found a significant difference between serum IL-8 levels in the dogs with non-metastatic tumours and those with metastatic tumours. However, in this study the group with metastatic tumours consisted of only 3 animals. In turn, Estrela-Lima *et al.* (2013) have observed lower plasma levels of IL-8 in dogs with metastasis than in the animals without metastasis.

An increased plasma levels of IL-1 α and IL-8 in the dogs with metastatic tumours, found in the present research and in other studies, may indicate that in the microenvironment of metastatic tumors greater amount of these cytokines are produced than in non-metastatic tumours. It is known that both tested cytokines can be produced in the microenvironment of tumour by the tumour cells, tumour-infiltrating immune cells as well as stromal cells (Raman *et al.*

2007, Fasoulakis *et al.* 2018, Malik and Kanneganti 2018). Studies involved animal model of breast cancer and breast cancer cell lines have shown that the metastatic breast cancer cells produce higher levels of IL-8 compared to the non-metastatic cells (Todorović-Raković and Milovanović 2013). Another explanation could be a size of the metastatic tumours. All metastatic tumours found in the present study were over 5 cm in size. The large tumours comprised many tumour cells and inflammatory cells, which can result in the high plasma/serum cytokine levels (Raman *et al.* 2007). Monkong *et al.* (2020) have reported an increase in the serum IL-8 concentration with increasing canine mammary tumour size. Similarly, Gelaleti *et al.* (2012) as well as Haas *et al.* (2015) have found a relationship between the serum IL-8 concentration and the tumour size.

Our findings showed that IL-1 α and IL-8 concentrations increased in the dogs with grade 3 mammary carcinomas compared to the animals with grade 1 and grade 2 carcinomas. This may suggest that the increased level of these cytokines is responsible for transforming tumour cells into a more malignant phenotype. Another explanation could be the greater production of the cytokines in more malignant tumours. In contrast, Machado *et al.* (2015) have found no significant difference in the serum IL-1 concentration between low-grade and high-grade canine mammary tumours. In turn, the study by Haas *et al.* (2015) has shown that the serum IL-8 concentration in dogs with neoplastic disease decreases with raising malignancy (but differences were not statistically significant). Studies in humans have shown that the increased serum IL-8 concentration was associated with higher malignancy of various cancers, including breast cancer (Yokoe *et al.* 1996, Benoy *et al.* 2004). Another study has shown that the high expression of IL-1 α in breast cancer biopsies was associated with more malignant phenotypic behaviour (Singer *et al.* 2006).

It is believed that IL-1 α signaling in the tumour tissue and its microenvironment can promote tumour progression in a variety of ways. As an important pro-inflammatory cytokine, IL-1 α induces potent inflammatory responses at tumour sites that leads to an increased invasiveness of the malignant cells (Tjomsland *et al.* 2011). Tumour-derived IL-1 α , acting on infiltrating myeloid cells, induced the expression of a critical tumour survival factor, the cytokine TSLP (Thymic Stromal Lymphopoietin). TSLP promotes the survival of the tumour cells through induction of the expression of the anti-apoptotic molecule Bcl-2 (Kuan and Ziegler 2018). Moreover, IL-1 α increases tumour cell proliferation and promote angiogenesis (Salven *et al.* 2002, Chiu *et al.* 2021). On the other

hand, some studies have shown anti-tumorigenic role of IL-1 α (Maund et al. 2011, Maund et al. 2013).

IL-8 may promote tumour growth and survival through its mitogenic and angiogenic properties (Benoy et al. 2004, Yoan et al. 2005, Todorović-Raković and Milovanović 2013). It has been shown a stimulating effect of IL-8 on the expression of the vascular endothelial growth factor (VEGF) in endothelial cells, and the production of matrix metalloproteinase MMP-2 and MMP-9 by tumour cells (Li et al. 2005). The secreted IL-8 stimulates the expression of the adhesion molecule fibronectin in human breast carcinoma cells and the induction of motile phenotype of these cells (Mohamed 2012). It has been also found that cyclooxygenase-2 increases the invasiveness of breast cancer through IL-8 activation (Simeone et al. 2007).

In conclusion, the results of our study demonstrate that plasma IL-1 α and IL-8 concentrations are increased significantly in the dogs with malignant mammary tumours and they are significantly higher in cases of metastatic tumours than in non-metastatic tumours. Moreover, high concentrations of these cytokines are associated with the high tumour grade. These findings may also indicate that an increased malignancy and invasiveness of canine mammary tumours is associated with an increased production of these cytokines in the tumour microenvironment, which, in turn, leads to an increase in their circulating levels. This suggests that circulating levels of IL-1 α and IL-8 could be considered as diagnostic and prognostic markers in canine malignant mammary tumours. However, further studies in this fields are needed.

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