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RESEARCH ARTICLE

Comparison of Plasma Interleukin-6 and Interleukin-10 Concentrations between Female Dogs with Mammary Gland Tumours and Healthy

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A B S T R A C T

The aim of this study was to determine the concentrations of IL-6 and IL-10 in plasma of female dogs with benign and malignant mammary gland tumours and comparison to that in healthy. The study was carried out on 44 dogs, including 34 dogs with mammary gland tumours (8 benign tumours and 26 malignant tumours) and 10 healthy controls. The concentrations of tested cytokines were analysed using a specific canine ELISA assay. The study showed that both groups of dogs with mammary gland tumours had significantly higher ($P<0.05$) concentrations of IL-6 compared to control group. Similarly as in the case of IL-6, dogs with mammary tumours had increased concentrations of IL-10 compared to healthy dogs. Only in relation to dogs with malignant tumours a difference reached statistical significance ($P<0.05$). In dogs with mammary carcinomas, the IL-6 concentration increased with histological grade of tumours and dogs with grade 3 tumours had a significantly higher ($P<0.05$) concentration of IL-6 than those with grade 1 tumours. Although the highest concentration of IL-10 was observed in dogs with grade 3 tumours significant differences were not found. The strong correlation ($r=0.97$) was found between plasma IL-6 and IL-10 concentrations in relation to the health status of animals. Our results suggest that IL-6 and IL-10 are important factors involved in a neoplastic process in the mammary gland of dogs. In addition, the results obtained indicate that circulating IL-6 and IL-10 concentrations could be helpful for identifying of malignant form of mammary gland tumours in dogs.

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INTRODUCTION

There is strong evidence that tumour development and progression can be controlled by the immune system and that cytokines are involved in this process (Lin and Karin, 2007; Fernandes *et al.*, 2015). Cytokines can mediate both the mechanisms that inhibit tumour growth and promote tumour development and progression (Nicolini *et al.*, 2006; Newman and Gonzalez-Perez, 2014). It is known that a range of cytokines is produced and released in tumour microenvironment by inflammatory cells as well as by tumour cells and stroma (Ben-Baruch, 2003). It is believed that synthesis and release of cytokines in tumour microenvironment leads to increase of their circulating levels that 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).

Interleukin 6 (IL-6) belongs to pro-inflammatory cytokines and is produced by different inflammatory/ immune cells, as well as by tumour cells (Ben-Baruch, 2003; Kim *et al.*, 2010). There is much evidence that IL-6 promotes tumour development and progression due to its role in tumour cell proliferation, survival, angiogenesis, inflammation and metastasis (Nicolini *et al.*, 2006). The high serum level of IL-6 has been demonstrated in humans with different tumours, including breast cancer, and has been linked to poor patient outcome (Salgado *et*

al., 2003; Lyon *et al.*, 2007). It is suggested that IL-6 neutralization may be useful in the treatment of cancer in humans (Guo *et al.*, 2012).

Interleukin-10 (IL-10) is one of the anti-inflammatory cytokines that inhibits the production of pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6 and tumour necrosis factor alfa (TNFα) (Hamidullah *et al.*, 2012). In tumour microenvironment the source of this cytokine may be both inflammatory cells and tumour cells (Sabat *et al.*, 2010). It is believed that IL-10 mainly has anti-tumour activity, but it may also promote tumour growth (Hamidullah *et al.*, 2012). Increased serum IL-10 concentrations have been found in women with breast cancer and correlation has been observed between high levels of IL-10 and advanced clinical stage of the disease (Merendino *et al.*, 1996).

Mammary gland tumours, next to skin tumours, are the most common neoplasms in dogs (Sleeckx *et al.*, 2011), exhibiting similar traits to breast cancer in women in terms of epidemiological, morphological, pathological and biochemical features (Kumaraguruparan *et al.*, 2006). Although the role of IL-6 and IL-10 has been intensively studied in women with breast cancer and changes in circulating concentrations of these cytokines have been shown in breast cancer patients, the literature on serum/plasma concentrations of these cytokines in female dogs with mammary gland tumours is scarce. Thus, the aim of this study was to determine the concentrations of IL-6 and IL-10 in plasma of female dogs with benign and malignant mammary gland tumours and comparison to that in healthy.

MATERIALS AND METHODS

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

Animals and design of study: Forty four purebred or mixed-breed female dogs were used for this study including 34 dogs with mammary gland tumours (age ranging from 6 to 13 years) and 10 healthy controls (aged 3-8 years). The animals were patients of the Department of Animal Reproduction, Faculty of Veterinary Medicine in Lublin. The animals with mammary tumours were selected from the group of female dogs undergoing surgery due to spontaneously occurring mammary gland tumours. The control group undergoing sterilization at the owner's request. None of the female dogs had use of any drugs within 30 days prior to surgical procedure.

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

The surgical resection of mammary tumours was performed according to standard practice, with an aim to remove the tumour with complete margins. The sections of removed mammary tumours were fixed in 10% neutral buffered formalin for 24h, embedded in paraffin blocks and sliced into 4μm sections. The microscopic preparations, stained with haematoxylin and eosin (HE), were evaluated histologically according to the WHO classification of tumours and mammary gland dysplasia (Misdorp *et al.*, 1999). Malignant epithelial neoplasms were graded according to the Nottingham method for human breast tumours, adopted for canine mammary tumours (Elston and Ellis, 1998).

All female dogs with mammary tumours qualified for the study were diagnosed with primary, single, malignant tumour, 2.5 to 5.1cm in size, without inflammatory reaction and/or ulceration. Based on histological findings, animals were divided into two groups: (1) female dogs with benign mammary tumours (n=8) and (2) female dogs with malignant mammary tumours (n=26)

Nine millilitres of blood from all female dogs were collected from cephalic vein into Vacuette tubes immediately before surgery. The plasma obtained after centrifugation was immediately frozen to -76°C and kept deeply frozen until used for the determination of IL-6 and IL-10.

Laboratory analysis: The concentrations of IL-6 and IL-10 in the plasma samples were analyzed 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 450nm. The detection limit of canine IL-6 was 3.1 pg/mL. Intra- and inter-assay coefficients of variation were below 10 and 12%, respectively. The detection limit of canine IL-10 was 5.9 pg/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 one-way ANOVA test with the HSD Tukey's test was used to determine significant differences in the concentrations of IL-6 and IL-10 between the study groups. The correlations between IL-6 and IL-10 concentrations were presented using Pearson's correlation coefficient. Differences at $P<0.05$ were considered statistically significant.

RESULTS

Histopathological findings: Histopathological analysis showed that 8 dogs of the 34 dogs with mammary gland tumours had benign mammary tumours (6 complex adenomas and 2 benign mixed tumours). In 26 dogs diagnosed with malignant mammary gland tumours (2 *in situ* carcinomas, 9 complex carcinomas, 8 tubulopapillary carcinomas, 2 anaplastic carcinomas, 3 solid carcinomas, 1 osteosarcoma and 1 fibrosarcoma). The microscopic preparations of normal mammary gland tissues, benign mammary tumour (complex adenoma) and malignant mammary tumours (solid carcinoma and anaplastic carcinoma) are shown in Fig. 1. Among 24 malignant epithelial mammary gland tumours (carcinomas), 9 were grade 1 (G1) tumours, 10 were classified as grade 2 (G2) tumours and 5 were classified as grade 3 (G3) tumours.

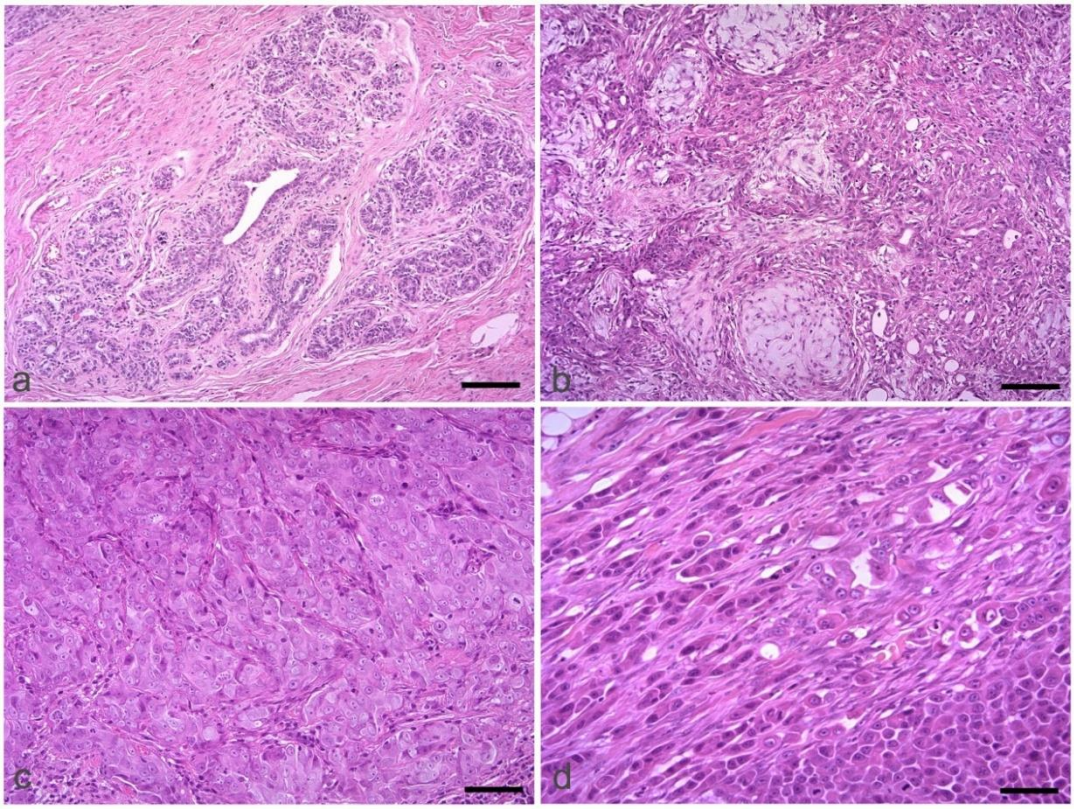

Fig. 1: Histopathological examination of mammary gland tumours in female dogs used in the study. (a) Normal mammary gland. The intralobular duct and secretory acini surrounded by loose connective tissue. HE. Bar = 100 µm, (b) Complex mammary adenoma. Compressed ducts surrounded by fusiform and stellate myoepithelial cells in a pale basophilic matrix. HE. Bar = 100 µm, (c) Simple solid mammary carcinoma, high grade. Highly pleomorphic, polygonal epithelial cells arranged in solid sheets without lumina. Note the high mitotic rate. HE. Bar = 50 µm, (d) Simple anaplastic mammary carcinoma, high grade. Individual neoplastic cells or small clusters of cells diffusely infiltrating reactive fibrous stroma. HE. Bar = 50 µm.

Plasma IL-6 concentrations: As shown in Table 1 both groups of dogs with mammary gland tumours had significantly higher ($P<0.05$) concentrations of IL-6 compared to control group. Although the concentration of IL-6 was numerically higher in dogs with malignant tumours than in those with benign tumours, a difference did not reach statistical significance. In plasma of dogs with malignant epithelial tumours (carcinomas), the mean concentration of IL-6 increased with tumour grade (G1-G3) and dogs with grade 3 tumours had a significantly higher ($P<0.05$) concentration of IL-6 than those with grade 1 tumours (Fig. 2). All these subgroups of female dogs had statistically significantly higher ($P<0.05$) concentrations of IL-6 than healthy dogs.

Plasma IL-10 concentrations: Similarly as in the case of IL-6, dogs with mammary tumours had increased concentrations of IL-10 compared to healthy dogs (Table 1). Only in relation to dogs with malignant tumours a difference reached statistical significance ($P<0.05$). The concentration of IL-10 was markedly higher in dogs with malignant tumours than in those with benign tumours; however, a significant difference was not found. When compared the mean IL-10 concentrations among dogs with different grade carcinomas (G1-G3), the highest value of IL-10 concentration was found in dogs with grade 3 tumours, but significant differences were not observed (Fig. 3). All dogs with carcinomas, irrespective of the tumour grade (G1-G3), had significantly higher concentrations of IL-10 compared to control group.

Table 1: Concentrations of IL-6 and IL-10 (mean±SD) in plasma of female dogs with mammary tumours and control

Parameter	Groups		
	Control (n=10)	Benign mammary tumours (n=8)	Malignant mammary tumours (n=26)
IL-6 (pg/mL)	1.84±0.1	12.05±3.74[a]	15.15±9.62[a]
IL-10 (pg/mL)	21.84±2.94	134.92±68.2	179.5±129.8[a]

[a]statistically significant differences ($P<0.05$) in comparison to control.

Correlation between plasma IL-6 and IL-10 concentrations: The correlation analysis showed the strong correlation ($r=0.97$) between plasma IL-6 and IL-10 concentrations in relation to the health status of animals (Fig. 4).

DISCUSSION

In contrast to numerous studies showing changes in the concentrations of various cytokines in serum/plasma of women with breast cancer, only a few reports are available with respect to circulating levels of cytokines in female dogs with mammary tumours. In this work the concentrations of IL-6 and IL-10 were investigated in plasma of female dogs with mammary gland tumours (benign and malignant) and healthy, using a specific canine ELISA assay. The results obtained indicated that the concentrations of these cytokines were higher in dogs with mammary tumours than in healthy dogs. Moreover, we found that dogs with malignant tumours had markedly higher levels of both cytokines than those with benign tumours. Both cytokines demonstrated increased concentrations in dogs diagnosed with grade 3 malignant

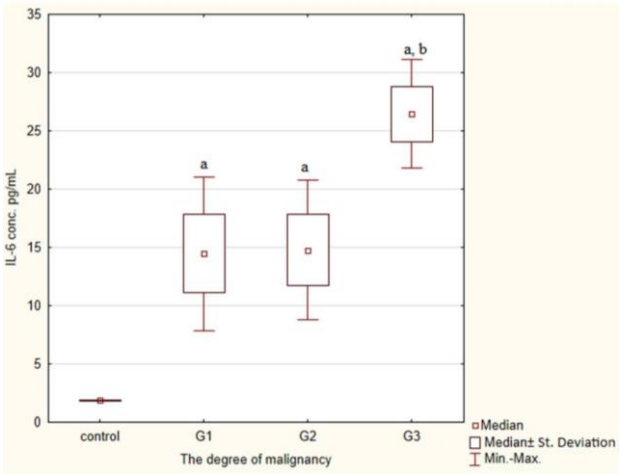

Fig. 2: Box plot showing plasma concentrations of IL-6 (pg/mL) in female dogs with various histological grade (G1-G3) mammary tumours. [a] – significant differences (P<0.05) in comparison to control group, [b] – a significant difference (P<0.05) in comparison to G1 group.

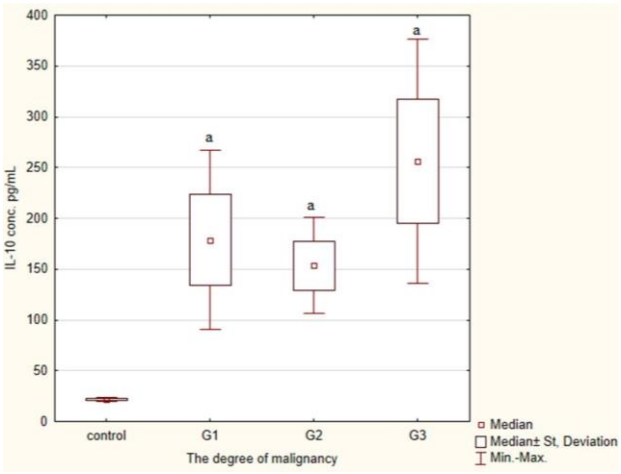

Fig. 3: Box plot showing plasma concentrations of IL-10 (pg/mL) in female dogs with various histological grade (G1-G3) mammary tumours. [a] – significant differences (P<0.05) in comparison to control group.

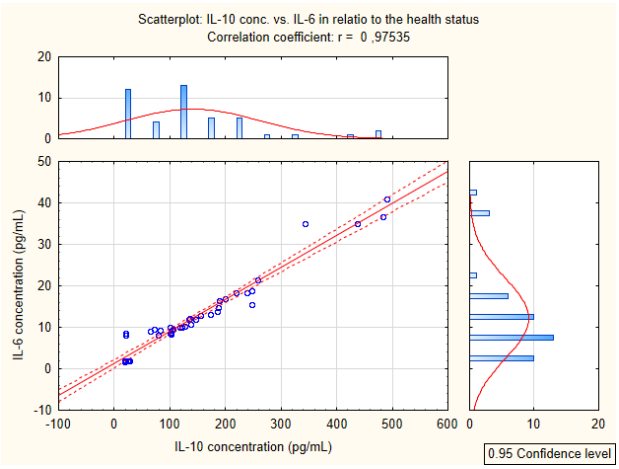

Fig. 4: Correlation between IL-6 and IL-10 plasma concentrations in female dogs with mammary tumours and healthy female dogs.

epithelial tumours (carcinomas) compared to dogs in which diagnosed tumours with lower grade, but significant differences were found only in terms of IL-6 concentrations. Our findings suggest that these cytokines are important factors in the development of canine mammary tumours.

Similar results were obtained by De Andres *et al.* (2013). These authors found significant differences in the serum concentrations of IL-6 between healthy dogs and those with benign tumours and inflammatory mammary carcinomas. In case of IL-10 a significant difference was found between dogs with inflammatory carcinomas and healthy controls. Many studies have shown increased serum/plasma levels of IL-6 (Zhang and Adachi, 1999; Lyon *et al.*, 2002; Salgado *et al.*, 2003) and IL-10 (Merendino *et al.*, 1996) in breast cancer patients. These findings support our results.

The fact that IL-6 concentration gradually increased from grade 1 mammary carcinomas to grade 3 carcinomas may suggest that this cytokine stimulates the transformation of the tumour to a more malignant form and indicate its potential value as diagnostic and prognostic marker. Martins *et al.* (2016) and Estrela-Lima *et al.* (2016) demonstrated a relationship between a high serum concentration of IL-6 and characteristics of canine malignant mammary tumours that reflect worse prognosis. In addition, immunohistochemical study showed an increased expression of IL-6 in malignant mammary tumours and tumours that metastasized compared to benign canine mammary tumours (Kim *et al.*, 2010).

IL-6 is a pro-inflammatory cytokine and can be produced by many cells of the immune system as well as by tumor cells (Ben-Baruch, 2003; Kim *et al.*, 2010). With regard to the impact on tumorigenesis, according to data from human medicine, IL-6 has mainly pro-carcinogenic activity. Il-6 can stimulate tumour growth by increasing proliferation and angiogenesis, and by impairment of the apoptotic process (Nicolini *et al.*, 2006; Guo *et al.*, 2012). Moreover, IL-6 increases the expression of estrogen receptors in the tumour (Purohit *et al.*, 2002). It was also shown that IL-6 may affect cell migration and promote metastasis formation (Asgeirsson *et al.*, 1998). On the other hand IL-6 may increase the activity of immune cells taking part in the defense against tumour (Nicolini *et al.*, 2006).

IL-10 exhibits both pro and anti-tumour activities, although it is believed that primarily inhibits tumour growth (Hamidullah *et al.*, 2012). IL-10 may exert anti-tumour effect by increasing the activity of immune cells that play an important role in the defense anti-tumour (Mocellin *et al.*, 2005). Moreover, IL-10 modulates apoptosis and suppresses angiogenesis during tumour regression (Hamidullah *et al.*, 2012). Another action of IL-10 that may reduce tumour growth is the inhibition of pro-inflammatory cytokine production, including TNFα and IL-6 (Lin and Karin, 2007). In contrast, some studies have indicated pro-tumour effect of IL-10 by the impairment of the immune defense against the tumour and increased angiogenesis (Pinzon-Charry *et al.*, 2005; Zeng *et al.*, 2010). In some human cancers IL-10 increased tumour cell resistance to apoptosis (Zeng *et al.*, 2010).

Some studies in women with breast cancer have shown that serum concentration of IL-10 depended on the tumour stage (Merendino *et al.*, 1996). In agreement with these studies, Estrela-Lima *et al.* (2016) demonstrated that dogs with mammary tumours that metastasized had elevated concentrations of IL-10 in serum compared to those without

metastasis. This is supported by study that showed a higher level of IL-10 in dogs with inflammatory mammary carcinomas than in dogs with non inflammatory mammary malignant tumours (De Andres *et al.*, 2013). These findings correspond with results of our study. We observed the highest mean plasma IL-10 concentration in female dogs with histological grade 3 mammary carcinomas. These results suggest that the high circulating levels of this cytokine may stimulate tumour cells to transform into a more malignant form and facilitate metastases.

Contrarily, Martins *et al.* (2016) demonstrated that a higher serum concentration of IL-10 in dogs was related to characteristics of malignant mammary tumours that reflect better prognosis, suggesting that IL-10 may play a protective role in these tumours. A protective role of IL-10 in relation to mammary tumours in dogs suggests also immunohistochemical study by Li *et al.* (2014) that showed a correlation between high IL-10 expression in canine mammary carcinomas and high overall survival.

Conclusions: The present study showed increased concentrations of both studied cytokines in plasma dogs with mammary tumours and markedly higher their concentrations in cases of malignant tumours than in cases of benign tumours. Moreover, the concentrations of these cytokines, especially IL-6, increased with increasing of histological grade of tumours. Our results suggest that IL-6 and IL-10 are important factors involved in a neoplastic process in the mammary gland of dogs. In addition, the results obtained indicate that circulating IL-6 and IL-10 concentrations could be helpful for identifying of malignant form of mammary gland tumours in dogs. Further studies are needed to investigate the diagnostic and prognostic value of circulating levels of these cytokines in female dogs with mammary gland tumors.

Authors contribution: MS conceived and designed the study. MS, MK and RD collected materials. MK and MB analyzed the blood samples. WŁ performed histopatological examination. RU performed statistical analysis. MS, MK and RD interpreted the data, critically revised the manuscript for important intellectual contents. All authors approved the final version.

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