Polish Journal of Veterinary Sciences Vol. 26, No. 3 (2023), 385–392

DOI 10.24425/pjvs.2023.145044

Original article

Plasma levels of chemokines CCL2 and CXCL12 in female dogs with malignant mammary gland tumours without and with metastases

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Abstract

The aim of this study was to evaluate the plasma levels of chemokines CCL2 and CXCL12 in female dogs with malignant mammary gland tumours without and with metastases. The concentrations of CCL2 and CXCL12 were determined in 25 female dogs with malignant mammary gland tumours (15 without metastases and 10 with metastases) and 10 healthy control animals using a specific canine ELISA assay. The mean plasma concentrations of CCL2 and CXCL12 were significantly higher ($p<0.05$) in the metastatic group compared to the control group. Moreover, the concentrations of these chemokines were markedly higher in the dogs with metastases than in those without metastases; however, a statistically significant difference was not found. The concentrations of both tested chemokines were numerically increased in the dogs with grade 2 and grade 3 carcinomas compared to the dogs with grade 1 carcinomas but the differences did not reach statistical significance. In conclusion, the results of our study demonstrate that plasma concentrations of chemokines CCL2 and CXCL12 are significantly increased in the dogs with metastatic malignant mammary gland tumours compared to the healthy dogs and show an upward trend compared to those without metastases. However, clarifying whether the increase of these chemokines is a cause or an effect of metastasis in female dogs with malignant mammary gland tumours as well as their potential role in metastatic process requires further research.

Keywords: canine mammary tumours, CCL2, chemokines, CXCL12, metastases, plasma

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Introduction

The behavior of cancer cells is highly influenced by networks of cytokines, of which a major role is played by the members of the chemokine superfamily (Kohli et al. 2022, Li et al. 2022). Many studies indicate that cancer cells and stromal cells in the tumour microenvironment, including endothelial cells, fibroblasts, mesenchymal stem cells and infiltrating leucocytes, produce various chemokines that fulfill numerous biological functions during tumour progression and metastasis (Lim et al. 2016, Gao and Fish 2018, Li et al. 2022). There is increasing evidence that especially chemokines CCL2 and CXCL12 are involved in the metastatic process (Lim et al. 2016, Gao and Fish 2018, Shi et al. 2020). Data from human medicine indicate that these chemokines significantly influence the biological behavior of various cancers, including breast cancer in women (Lim et al. 2016, Gao and Fish 2018, Shi et al. 2020, Zielińska and Katanaev 2020, Li et al. 2022).

Chemokine CCL2 (Monocyte Chemotactic Protein 1, MCP1) is a potent chemotactic factor for many immune cells, such as monocytes, dendritic cells, T-lymphocytes and natural killer cells (Deshmane et al. 2009, Li et al. 2022). CCL2 binding to its receptor (CCR2) triggers signal transduction pathways and as a result induces various biological effects (Lim et al. 2016). Studies on experimental models have shown that signaling pathways triggered by CCL2-CCR2 are especially important for successful metastasis, and are involved in all steps of the metastatic process (Lim et al. 2016, Li et al. 2022). It has been found that chemokine CCL2 is highly expressed in primary breast cancer cells and distant metastases but is expressed at a low level in healthy breast tissue (Soria and Ben-Baruch 2008). A significantly increased circulating concentration of CCL2 in patients with breast cancer has also been shown (Dehqanzada et al. 2007, Lubowicka et al. 2018). The concentration of CCL2 in these patients increasesd with the advancing tumour stage (Lubowicka et al. 2018). Moreover, in the breast cancer patients an increased level of CCL2 has been shown to correlate with lymph node metastasis and shorter overall survival (Yao et al. 2016).

Chemokine CXCL12 (Stromal Cell Derived Factor 1, SDF1) plays a critical role in monocyte migration and is both expressed under homeostatic conditions at sites where resident macrophages are present, and is highly inducible in some pathologic conditions involving ischemia and/or hypoxia, and in proangiogenic environments such as tumours (Sanchez-Martin et al. 2011, Janssens et al. 2018). Binding of CXCL12 to its receptor CXCR4 activates diverse signaling pathways that mediate, among others, tumour development and metastasis (Shi et al. 2020, Li et al. 2022). The CXCL12/CXCR4 signaling pathway has been found in recent years to be a key player in breast cancer tumorigenesis (Zielińska and Katanaev 2020). It is thought that this pathway controls many aspects of breast cancer development and progression, including cancer cell proliferation, cell survival, motility and metastasis to all target organs (Zielińska and Katanaev 2020). Significantly increased serum concentrations of CXCL12 have been found in women with breast cancer compared to healthy women (Aljurany and Samarrai 2022).

Malignant tumours account for over half of mammary gland tumours, the most frequently diagnosed tumours being in intact female dogs (Sleeckx et al. 2011, Salas et al. 2015). These tumours often metastasize to distant organs, mainly to the lungs, and consequently lead to the death of the animal (Sleeckx et al. 2011, Salas et al. 2015). Despite the fact that they constitute a serious clinical problem, the mechanisms involved in their development, progression and metastasis have not been fully understood. Considering the significance of CCL2 and CXCL12 for breast cancer, which shares many similarities with canine mammary gland tumours (Abdelmegeed and Mohammed 2018), it can be assumed that these chemokines also play an important role in canine malignant mammary gland tumours. However, data on the role of CCL2 and CXCL12 in canine tumours are very sparse, and to the authors' knowledge, there are no studies on the circulating levels of these chemokines in female dogs with malignant mammary gland tumours. Therefore, the aim of this study was to evaluate the plasma levels of CCL2 and CXCL12 in female dogs with malignant mammary gland tumours without and with metastases.

Materials and Methods

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

Animals and design of the study

Thirty five intact purebred or mixed-breed female dogs were used in this study including 25 dogs with malignant mammary gland (aged 6-13 years) and 10 healthy animals (aged 3-8 years) as controls. 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 a group of female dogs provided by owners for treatment due to spontaneously occurring mammary gland tumours. The control group consisted

of female dogs provided by owners for sterilization at the owner's request. All the owners gave informed consent to include their pets in the study.

Only female dogs with one mammary gland lesion recognized as a malignant tumour regardless of the tumour size or location, with no clinical signs of inflammatory mammary carcinoma and ulceration were qualified for the study. 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. Aspirated samples for cytology examination using the fine needle aspiration biopsy technique were acquired in cases of lymphadenopathy.

In the dogs with non-metastatic tumours, surgical resection of mammary tumours was performed according to standard practice, with the aim of removing 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.

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, urine determinations and abdominal ultrasound examinations were performed. All the animals in this group had no history of mammary tumours or other neoplasms, and were clinically healthy. None of the dogs used for the study had used 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 each of 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 CCL2 and CXCL12.

Laboratory analysis

The plasma concentrations of CCL2 and CXCL12 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 CCL2 was less than 6.6 pg/mL. Intra- and inter-assay coefficients of variation were below 10% and 12%, respectively. The detection limit of CXCL12 was less than 0.059 ng/mL. Intra-assay coefficients of variation were below 10% and inter-assay coefficients of variation were below 12%.

Statistical analysis

Statistical analysis was performed using STATISTICA version 10.0 (Statsoft, USA). The results were analysed for normal distribution using the Shapiro-Wilk test. The Kruskal-Wallis test followed by the Mann-Whitney test with the Bonferroni correction was applied to determine significant differences in the concentrations of CCL2 and CXCL12 between the study groups. Differences at $p<0.05$ were considered statistically significant.

Results

Clinical and histological characteristics of mammary gland tumours

The histopathological evaluation of mammary gland tumours from the female dogs used in the study showed that tubulopapillary carcinoma and complex carcinoma were the predominant histological types – 11 and 7, respectively. Three tumours were diagnosed as solid carcinomas, three as carcinosarcomas and one as an anaplastic carcinoma. Ten tumours metastasized (5 tubulopapillary carcinomas, 2 solid carcinomas, 2 carcinosarcomas and 1 anaplastic carcinoma). Eight tumors metastasized to the lungs and 2 tumours metastasized to regional lymph nodes and lungs. Among 22 malignant epithelial mammary gland tumours (carcinomas), 7 were grade 1 (G1) tumours, 9 grade 2 (G2) tumours and 6 grade 3 (G3) tumours. In the group of metastatic tumours histological grade 2 was found in 3 tumours and grade 3 in 5 tumours. Nine tumours were 5 cm or less in size and 16 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).

Table 1. Mean plasma concentrations of CCL2 and CXCL12 in female dogs with malignant non-metastatic and metastatic mammary gland tumours, and in the healthy animals (control).

Group	Number of dogs	CCL2 (pg/ml)	CXCL12 (ng/ml)
Non-metastatic	15	321.89 ±211.56	2.59±2.07
Metastatic	10	517.46±329.13[a]	5.71±2.93[b]
Control	10	154.21±127.86[a]	0.96±0.61[b]

[a, b] – the same letters mean statistically significant differences at $p<0.05$.

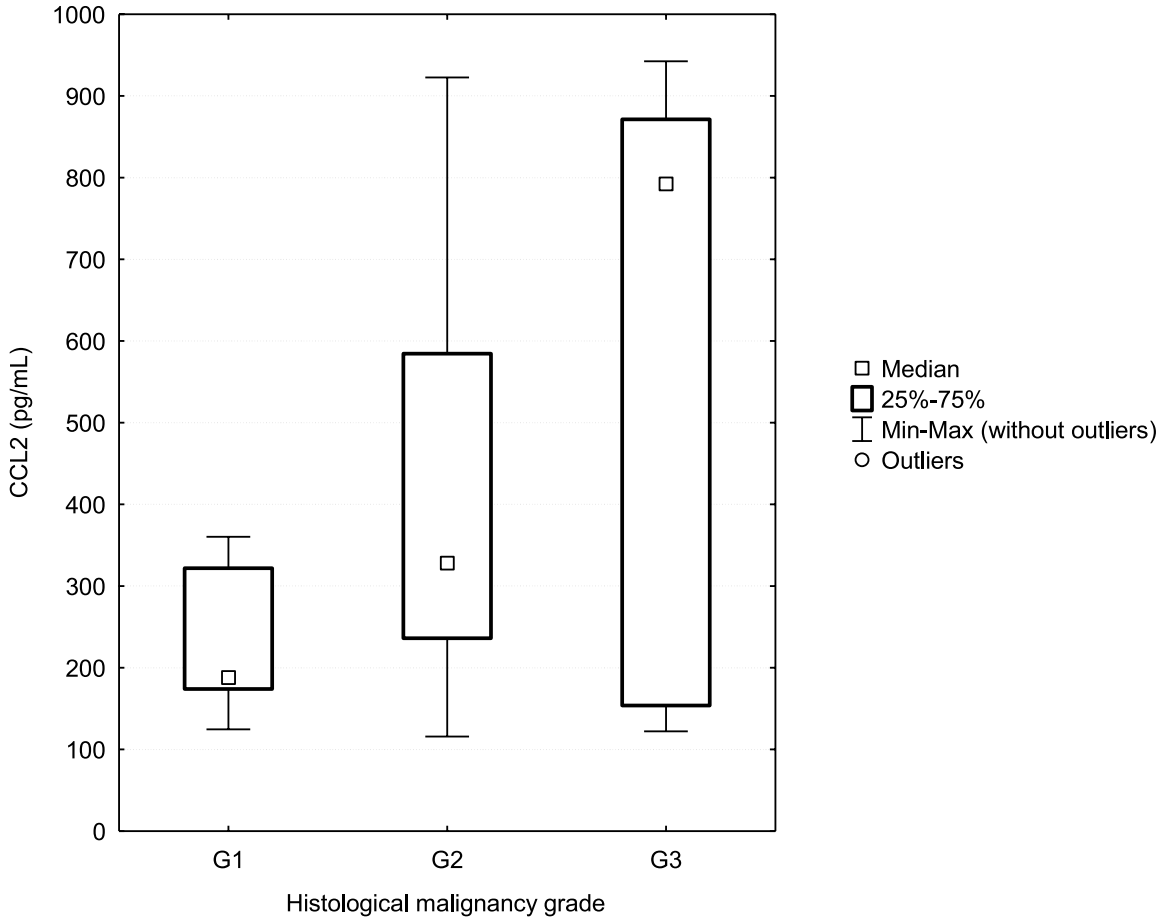

Fig.1. Plasma concentrations of CCL2 (pg/mL) in female dogs with various histological grade (G1-G3) malignant epithelial mammary gland tumours (carcinomas).

Plasma CCL2 concentration

The mean plasma concentration of CCL2 was markedly higher in the female dogs with malignant tumours compared to the control group; however, a significant difference ($p<0.05$) was only found between the control group and the metastatic group (Table 1). In the dogs with metastatic tumours, the concentration of CCL2 was numerically higher compared to the dogs with non-metastatic tumours but a significant difference was not found. The comparison of the concentrations of CCL2 in the dogs with various histological grades of epithelial malignant mammary tumours (carcinomas) showed no statistically significant differences, although the mean concentrations of CCL2 in the dogs with grade 2 and grade 3 tumours were markedly higher compared to dogs with grade 1 tumours (Fig. 1).

Plasma CXCL12 concentration

The mean plasma concentration of CXCL12 was significantly higher ($p<0.05$) in the female dogs with metastatic malignant mammary tumours compared to the healthy female dogs (Table 1). Although the concentration of CXCL12 was numerically higher in the metastatic group than in the non-metastatic group a significant difference was not found. Statistical analysis showed no significant differences in the concentrations of CXCL12 between the dogs with various grade carcinomas (Fig. 2).

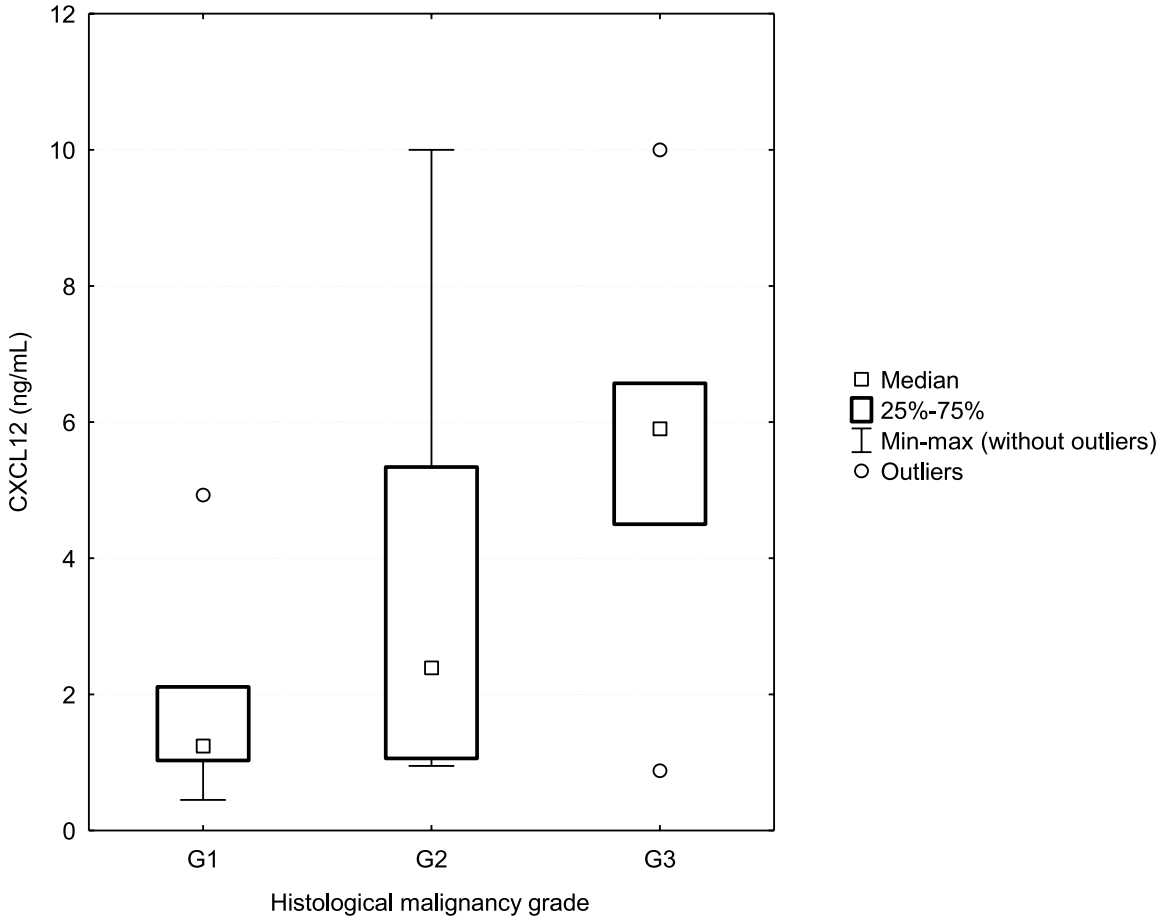

Fig. 2. Plasma concentrations of CXCL12 (ng/mL) in female dogs with various histological grade (G1-G3) malignant epithelial mammary gland tumours (carcinomas).

Discussion

In this study we measured the levels of chemokines CCL2 and CXCL12 in the plasma of female dogs with non-metastatic and metastatic malignant mammary gland tumours and healthy female dogs. To the authors knowledge, this is the first study regarding the circulating levels of these chemokines in female dogs with malignant mammary gland tumours.

In general, the concentrations of both tested chemokines were higher in the plasma of the female dogs with malignant mammary tumours than in the healthy female dogs; however, significant differences were only found in relation to the metastatic group. Furthermore, the plasma concentrations of these chemokines were markedly higher in the female dogs with metastatic tumours than in those without metastasis but the differences did not reach statistical significance. So far, very little research has been done on the determination of CCL2 and CXCL12 in female dogs with malignant mammary gland tumours. With regard to CCL2, significantly higher serum concentrations of this chemokine have been found in dogs with other malignant tumours. Ishioka et al. (2013) analyzed the level of CCL2 in the serum of 39 dogs with various neoplasms, including lymphoma, mastocytoma, thyroid carcinoma and gastric carcinoma. They found significantly higher concentrations of CCL2 in dogs affected with neoplasia than in healthy dogs. Other authors have observed significantly increased serum concentrations of CCL2 in dogs with pulmonary metastatic oral malignant melanoma (Maekawa et al. 2022), urothelial carcinoma (Shimizu et al. 2019), lymphoma (Perry et al. 2011) and histiocytic sarcoma (Nielsen et al. 2013). Allende et al. (2020) reported an increased serum level of CCL2 in dogs with osteosarcoma; however, a significant difference was not found between the affected dogs and the healthy controls. Moreover, an immunohistochemical study has indicated a significantly higher expression of CCL2 in metastatic canine malignant mammary tumours compared to healthy mammary gland tissue, benign tumours and non-metastatic malignant mammary tumours (Rybicka et al. 2016). In accordance with the results presented in this study, many studies in humans have shown significantly increased serum levels of CCL2 in patients with various cancer types, including breast cancer (Hefler et al. 1999, Wu et al. 2013, Pan et al. 2016, Lubowicka et al. 2018, Feng et al. 2020).

Moreover, the concentrations of CCL2 were significantly higher in patients with metastatic tumours than in those without metastases (Lu et al. 2011, Lubowicka et al. 2018).

The results obtained regarding the determination of CXCL12 are supported by the findings of Ariyarathna et al. (2020). These authors found a significantly higher expression of gene CXCL12 in canine mammary tumours which subsequently metastasized than in tumours that did not metastasise. The results of our study are also consistent with the results from feline mammary carcinoma studies (Marques et al. 2017, Marques et al. 2018). These studies have shown higher serum CXCL12 concentrations in cats with mammary carcinoma than in healthy controls (Marques et al. 2017). In addition, metastatic feline malignant mammary tumours have shown a high expression of CXCL12 in the metastatic lesions located in the liver and lung (Marques et al. 2018). Studies on breast cancer have reported a significantly increased serum concentration of CXCL12 in women with this cancer compared to healthy women (Aljurany and Samarrai 2022). A stimulating effect of CXCL12 on breast cancer cell migration to lymphatic networks has also been found (Hayasaka et al. 2021).

A significant increase in the plasma concentrations of CCL2 and CXCL12 in female dogs with metastatic malignant mammary gland tumours was probably the result of their increased production in these tumours. It is known that both chemokines can be produced by the cancer cells and stromal cells in the tumour microenvironment (Orimo et al. 2005, Deshmane et al. 2009, Lim et al. 2016). It is suggested that an increased production of these chemokines in patients with neoplasia may be mediated by inflammatory cytokines, such as TNF α and interleukins produced by the tumour cells (Ferrari et al. 2018). Another explanation could be the size of the metastatic tumours. All metastatic tumours found in the present study were over 5 cm in size. Large tumours comprise many tumour cells and inflammatory cells, which can result in high circulating levels of chemokines (Raman et al. 2007). In addition, the presence of metastases could increase the total weight of the tumour tissue and thus the number of chemokines produced. The concentration of the tested chemokines could also be affected by the intensity of the inflammatory reaction in the tumours. In the present study all tumours showed no clinical signs of inflammation and ulceration; however, the presence of inflammatory infiltrates in the tumours was not assessed. Further studies on larger and more diverse materials are necessary to evaluate the influence of various clinical and histological features of canine malignant mammary tumours on circulating levels of CCL2 and CXCL12.

High levels of CCL2 and CXCL12 in dogs with metastases may also suggest that both chemokines are associated with the metastatic process of canine malignant mammary gland tumours. It is thought that CCL2 and CXCL12 are important factors in the metastasis of breast cancer in women (Lu et al. 2009, Qian et al. 2011, Lim et al. 2016, Zielińska and Katanaev 2020, Hayasaka et al. 2021). It has been suggested that increased levels of these chemokines in the tumour microenvironment may increase an influx of inflammatory cells into the tumour (Lubowicka et al. 2016, Zielińska and Katanaev 2020, Kohli et al. 2022). Consequently, tumour behaviour changes in a way that promotes tumour growth and spread. CCL2 and CXCL12 promote cancer cell survival and their migration by interacting with their receptors (CCR2 and CXCR4, respectively) expressed on tumour cells (Tang and Tsai 2012, Lim et al. 2016, Shi et al. 2020, Zielińska and Katanaev 2020). *In vitro* studies indicate that CXCL12 stimulates breast cancer cell migration to lymphatic networks (Hayasaka et al. 2021) and promotes the migration and invasion of canine hemangiosarcoma cells (Im et al. 2017). Moreover, CCL2 and CXCL12 induce the expression of metalloproteinases in cancer cells, increasing invasion (Tang and Tsai 2012, Lim et al. 2016, Shi et al. 2020). It has been found that both CCL2 and CXCL12 promote angiogenesis and thus tumour growth (Soria and Ben-Baruch 2008, Rybicka et al. 2016, Gao and Fish 2018, Shi et al. 2020). CXCL12 stimulates epithelial-to-mesenchymal transition in the tumour cells, which leads to loss of cell adhesion (Li et al. 2022).

In the present study the plasma concentrations of CCL2 and CXCL12 tended to be higher in the groups of dogs with grade 2 and grade 3 carcinomas compared to those with grade 1 carcinomas; however, a statistically significant difference was not found. These findings may suggest that in the case of canine malignant epithelial mammary tumours a circulating level of CCL2 and CXCL12 is not correlated with the histological malignancy of the tumour. Our results are consistent with the results of the study by Ariyarathna et al. (2020). These authors identified no significant differences in chemokine gene CXCL12 expression between different histological types and histological grades of canine malignant mammary tumours. In contrast, a human study has shown a significant increase in serum concentration of CCL2 with increasing histological grade of ovarian cancer (Hefler et al. 1999).

In conclusion, the results of our study demonstrate that plasma concentrations of chemokines CCL2 and CXCL12 are significantly increased in dogs with metastatic malignant mammary gland tumours compared to healthy dogs, and show an upward trend compared

to those without metastases. However, clarifying whether the increase of these chemokines is a cause or an effect of metastasis in female dogs with malignant mammary tumours, as well as their potential role in the metastatic process, requires further research.

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