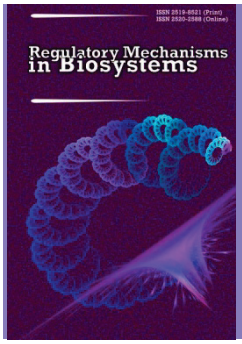

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst.,
2021, 12(4), 649–654
doi: 10.15421/022189

Prognostic markers of canine mammary tumours: Retrospective study of 142 cases

M. S. Kovalenko*, D. D. Bilyi*, P. M. Skliarov*, S. N. Maslikov*,
N. I. Suslova*, T. L. Spitsyna*, I. D. Yevtushenko**

**Dnipro State Agrarian and Economic University, Dnipro, Ukraine*
***State Biotechnological University, Kharkiv, Ukraine*

Article info

Received 08.09.2021
Received in revised form 03.10.2021
Accepted 04.10.2021

Dnipro State Agrarian and Economic University, S. Efremov st., 25, Dnipro, 49600, Ukraine. Tel.: +38-050-866-45-75. E-mail: klinika_best@i.ua

State Biotechnological University, Alchevskikh st., 44, Kharkiv, 61002, Ukraine. Tel.: +38-066-298-22-99. E-mail: aevt76@gmail.com

Kovalenko, M. S., Bilyi, D. D., Skliarov, P. M., Maslikov, S. N., Suslova, N. I., Spitsyna, T. L., & Yevtushenko, I. D. (2021). Prognostic markers of canine mammary tumours: Retrospective study of 142 cases. Regulatory Mechanisms in Biosystems, 12(4), 649–654. doi:10.15421/022189

Due to relevance of the problem, prediction of biological behaviour of neoplasias in mammary glands of dogs requires using contemporary approaches to the study, first of all, of ways of dissemination of tumour cells. One of them is studying the mechanisms of migration of cancer cells out of the neoplasm tissues with further dissemination and development of metastatic sites in the regional lymphatic nodes and remote tissues. We studied the survival period of bitches with tumours of the mammary glands following regional or unilateral mastectomy. Among malignant mammary tumours in bitches, the most often diagnosed were single tumours (57.5%), which histologically were classified to carcinomas – ductal (26.9%) and mixed type (21.9%). Probability of intratumoral invasion to blood vessels equaled 12.0%, to lymph vessels – 7.8%, lymph nodes – 12.8%. It depends on the histological type of the tumour, the most aggressive potentially being comedocarcinoma, tubulopapillary carcinoma and ductal carcinoma. Parameters of life expectancy and survival level after mastectomy depend on clinical stage of the disease (increase in the stage from the first to the third was characterized by decrease from 12.8 ± 9.5 to 9.4 ± 7.8 months), presence of angio/lymphatic invasions, presence of angiolymphatic invasion, but had no correlation with the size of the tumours. An important predictor of tumour-related death of dogs suffering neoplasias of the mammary glands is index vet-NPI, which has significant correlation with the clinical stage according to Owen and median survival. In particular, median survival in patients with the index lower than 4 exceeded the corresponding values in dogs with the index above 4 by 1.3 times. A promising direction of further research would be studying biological mechanisms of development of tumour emboli in the blood and lymph vessels, metastatic sites in lymph nodes, and also determining their role in pathogenesis of canine mammary tumours.

Keywords: dogs; neoplasias of mammary glands; lymphatic invasion; vascular invasion; survival.

Introduction

Despite the significant progress in diagnostics and treatment that has been achieved over the recent several decades, mammary gland tumours remain the global cause of early death of people and veterinary patients (Mysak et al., 2018; Terajima et al., 2021). Comparative studies of the mammary gland tumours in dogs, which were conducted in different countries, should take into account possible change in the prediction due to different shares of sterilized females among the general population of dogs (Peña et al., 2013).

Canine mammary tumours are heterogenic diseases classified into sarcomas, carcinomas and carcinosarcomas according to the histopathologic differentiation (Turna et al., 2021); they are characterized by different biological behaviours (Im et al., 2014). Mixed mammary tumours are the commonest type of tumours in the mammary gland of bitches; this form of neoplasia is associated with better predicted outcome than malignant tumours of other histological types (Cassali et al., 2012).

As of now, most studies on determining the level of aggressiveness of tumours of the mammary gland in dogs are related to studying correlative relations between relapses and metastases and age, clinical parameters and hormonal imbalance. At the same time, the role of invasion of tumour cells into blood and lymph vessels as predictor of disease course and efficiency of treatment is practically unanalyzed. In a multi-center retrospective study, Burrai et al. (2020) demonstrated that the most significant factors of the prognosis are the size of the tumour and age of the animal. Presence of metastases in the regional lymph nodes is an important factor influencing the prediction and treatment in cases of mammary tumours (Collivignarelli et al., 2021). Yamagami et al. (1996) observed no significant differences in the survival of bitches with different subtypes of adenocarcinomas (tubular, papillary and cystic papillary). In the conditions of multiple tumours of the mammary gland, average and overall sizes of malignant tumours are related to non-relapse life (Pecile et al., 2021). The level of methylation of DNA chains (evaluated by 5-methylcytosine) in mammary tumours correlates with size and prognosis for patients (Biondi et al., 2021).

Expression of Ki-67 in tumours of the mammary glands in dogs with overweight is higher than in dogs with normal weight (Nicchio et al., 2020). Higher body weight index is related to greater distribution of more aggressive mammary tumours and negatively affects the life expectancy of bitches with mammary tumours (Costa-Santos et al., 2019; Tesi et al., 2020).

Tumour-associated stroma influences the progression of tumours, including carcinoma of the mammary tumours of dogs (Amini et al., 2020; Pöschel et al., 2021). The tissues of malignant neoplasias of the mammary tumours of dogs have longer and wider collagen fibers compared with non-tumour and benign changes. In samples of malignant tumours of the mammary gland, lysine residuals, especially telopeptides, are significantly more hydroxiled compared with non-tumour tissues of the mammary gland. Such changes promote increase in mediated transversal binding of collagen in the conditions of neoplasias in the mammary gland, which may cause metastasis of cancer cells in such patients (Terajima et al., 2021). In different histological types of tumours of the mammary gland in canines, there were observed different patterns of collagen fibers: fibers in tubular carcinoma were located mainly around its parenchyma, while in solid tumour, they were across the entire parenchyma and around each

tumour cell (Borghesi et al., 2021). Sex hormones (estradiol for example), which are important in carcenogenesis of the mammary gland, can cause initiation and progression of tumours (Torres et al., 2021). Progesteron receptors play a key role in the development of mammary tumours in dogs, antiproliferative effects of aglepristone during mammary carcinomas in dogs are related to isoform of the progesterone A receptor (Guil-Luna et al., 2014). Membrane component 1 of progesterone receptors expresses less intensely in canine mammary tumours (Terzaghi et al., 2020). Mammary tumours lose oxytocin receptors proportionately to their malignancy (Benavente et al., 2019). Expression of estrogen receptors does not correlate with p53 protein, but is negatively associated with proliferation marker Ki-67; in the conditions of estrogen-positive tumours of the mammary glands, survival was higher compared with estrogen-negative tumours (Brunetti et al., 2021). Contrary to mammary tumours in women, excessive expression of HER-2 in dogs is not a factor for an unfavourable prognosis (Ressel et al., 2019).

P-glycoprotein and breast cancer resistance protein (BCRP) are the main compounds that cause the phenomenon of poly-resistance of mammary gland carcinomas in humans and dogs; those substances expressed in 52.0% and 74.5% of mammary tumours in dogs, respectively (Levi et al., 2021) and 85% and 80% of inflammatory carcinomas of the mammary gland (Levi et al., 2019). Expression of heat shock proteins correlated with malignancy of breast tumours in dogs (Okada et al., 2021). There is a correlation between the expression of COX-2 and high average value of mitotic index in tumors of the mammary gland in bitches (Badowska-Kozakiewicz & Malicka, 2010). At the current stage, predicting biological behaviour of mammary tumours in dogs is complicated, because the generally accepted TNM system is adapted only to singular tumour nodes. Moreover, for multiple tumours, the TNM system is limited because choosing which tumour is representative is arbitrary.

The objective of the study was determining the main factors for prognosis of spontaneous tumours of the mammary glands in dogs that lived in the conditions of anthropogenic pollution.

Materials and methods

The study was carried out according to the requirements of the "European Convention for Protection of Vertebrate Animals used for Scientific Purposes" (Strasburg, 1986) and the Law of Ukraine "On Protection of Animals from Abuse" (2006), as confirmed by the conclusions of the Committee of Bioethics of the Dnipro State Agrarian-Economic University. The owners had given permission for surgical treatment and also use of the material for scientific purposes.

A group of female dogs that had been diagnosed spontaneous mammary tumours in January 2017 – May 2021 at Best veterinary clinic in Zaporizhia (Ukraine) were admitted to the study retrospectively. Dogs that were included in this study received surgical treatment only. At the same time, using clinical and special (X-ray, ultrasound diagnostics, determining hemostasiologic status) methods of studies, we excluded presence of metastatic sites in other organs and tissues in such patients. The clinical stage of tumour process was determined according to Owen's TNM classification. This study included dogs with stages I–III of cancer.

At the first stage, tumours of the mammary gland of bitches were removed using the electric-surgical method during regional or unilateral mastectomy. We cut out the pieces from the tumour node(s), undamaged tissue of the mammary gland and regional lymph nodes: axillary and additional lymph nodes – in the conditions of damage to milk sacs I or II, axillary – damages IV or V, vaginal, additional or groin – in conditions of cancer III of the milk sac. To facilitate the identification of lymph nodes in the surgical material 7–10 minutes before surgery in the areas of milk sacs I or V, we subcutaneously injected 0.2–0.4 mL of 0.05% aqueous solution of methylene blue. Pieces of the tissues were fixated for 48–72 h in 10% neutral buffered formalin according to Lillie. Further, the tissues were passed through alcohols of ascending concentrations, chloroform, chloroform-paraffin and paraffin and embedded in paraffin. We made 5–6 μm-thick sections on the rotation microtome MPS-2 (Mikromed, Ukraine). Paraffin-embedded sections were deparaffinated in xylene and stained hematoxylin and eosin. The microscopy was carried out using an Olympus BX43 microscope (Olympus, Japan). Tumours were histologically verified according to the classification of Goldschmidt et al. (2011). Histological grading was determined based on the criteria of Elston & Ellis (1991) in the modification of Peña et al. (2013). Vascular invasion was determined as presence of tumour emboli in the intersections covered by endothelium in lymph or blood vessels. Lymph node was considered positive in relation to metastatic lesion if one or more groups of two or more atypical, non-lymph and non-haemopoietic cells were present inside the node. Furthermore, the Nottingham prognostic index (Haybittle et al., 1982) was adapted for canine mammary tumours by Santos et al. (2015) and is determined as Nottingham prognostic index adapted to veterinary medicine; it (hereinafter Vet-NPI) was calculated as size of tumour [cm]×0.2 + malignancy degree (1, 2, or 3) + signs of invasion to vessels or metastases into regional lymph nodes (1 or 2, respectively, if they were absent or present).

We examined 142 bitches with malignant mammary tumours aged 4 to 15 years (mean – 9.2 years). The number of cases identified to each histological subtype is presented in Table 1. Multiple tumours were diagnosed in 60 out of 142 (42.5%) cases. Cases that corresponded to histotype criteria of inflammatory carcinoma were excluded from the research.

Overall survival was calculated from the date of diagnosis to date of death/euthanasia of animal due to neoplastic disease. Animals that died or were euthanized due to causes unrelated to the mammary tumour and those lost for further monitoring were censored respectively at the moment of death or their last clinical monitoring.

Overall survival was determined employing Kaplan–Meier estimator, with test of logarithmic scale (Mantel–Cox) to estimate the differences between the shares of survival time. To determine the sensitivity and specificity of vet-NPI and clinical stage for the prognosis of the general survival, we used ROC-curve. Correlation analysis was performed according to Pearson. Statistical significance was determined at the level of 5%. All the analyses were carried out using Statistica 10 (StatSoft, Inc., USA). Sensitivity and specificity were determined using ROC-analysis with SPSS Statistics, version 24 (SPSS, Inc., IBM Company, Chicago, Illinois).

Results

Most often, malignant tumours were ductal carcinomas and carcinomas of mixed type – 38/142 (26.9%) and 31/142 (21.9%) respectively. At the moment of diagnosis, 12.0% (17/142) of malignant tumours demonstrated intratumoral vascular invasion, and 7.8% (11/142) demonstrated lymphatic invasion. Metastatic damage to lymph nodes was observed in 12.8% (18/142) of cases. Of 141 cases, 35 of 142 (24.8%) deaths were due to progressing disease during the monitoring (autopsy was performed in 4 cases and common metastatic disease was confirmed histologically), 79 of 142 (56.0%) patients lived 6 months after surgery, 52 of 142 (36.8%) lived 12 months, and 25 of 142 (17.7%) lived 18 months.

Analysis of probabilities of development of tumour emboli in vessels and regional lymph nodes (Table 1) indicates that incidence of malignant mammary growths significantly varies depending on histological type. In particular, angioinvasion was determined: for comedocarcinoma – in 50.0%, tubulopapillary carcinoma – 20.8%, ductal carcinoma – 10.1%, carcinoma-mixed type – 6.5% of the cases; lymphoinvasion – 25.0%, 20.8%, 5.1%, 6.5% of animals respectively, presence of metastases in regional lymph nodes – 50.0%, 20.8%, 12.8%, 13.0% of patients, respectively. During tubular carcinoma, there were only determined angio- and lymphoinvasion – in 40.0% and 20.0% of bitches against the background of absence of lesions in lymph nodes, while carcinoma-simple type – emergence of tumour nodes in blood vessels and lymph nodes of 9.5% of dogs. Metastatic sites in lymph nodes against the background of mucinous carcinoma did not allow us to evaluate the aggressiveness of this type of neoplasia, because only a singular case was found.

At the same time, tumours of the mammary gland such as carcinoma *in situ*, cribriform carcinoma and spindle cell carcinoma were not accompanied by migration of cancer cells out of the neoplastic tissue.

Cases of carcinomas *in situ* have much better prognoses compared with all other subtypes (P = 0.05). None of the animals with carcinoma *in situ* died of neoplastic disease (Fig. 1a). By contrast, all 4 animals with comedocarcinoma died of disease-related causes, and this subtype had shorter mean life expectancy (on average 2.7 months; 95% CI – 1–

7 months) compared with all other subtypes (mean values – 9.6 months; 95% of CI – 0–39 months; P = 0.0002) (Fig. 1b). In cases of cribriform carcinoma, 5 of 6 animals died of progression of the disease, but with higher average survival time (mean values: 9 months; 95% of CI, 3–15 months; P = 0.00031, Fig. 1c). Average survival time of animals suffering tubulopapillary carcinoma was statistically higher than in cases of tubular carcinoma (mean values – 11.7 months against 6.5 months; 95% of CI, 0–35 months and 1–22 months respectively; P = 0.005, Fig. 1d).

Table 1

Vascular invasion and lymphatic nodes involvement in various histological types of breast tumours in a cohort of 142 bitches

Type	Angioinvasion, %	Lymphoinvasion, %	Lymph node invasion, %
Carcinoma *in situ*(n = 10)	0.0	0.0	0.0
Carcinoma mixed type (n = 31)	6.5	6.5	13.0
Carcinoma simple (n = 21)	9.5	0.0	9.5
Comedocarcinoma (n = 4)	50.0	25.0	50.0
Cribriform carcinoma (n = 6)	0.0	0.7	0.0
Ductal carcinoma (n = 39)	10.1	5.1	12.8
Mucinous carcinoma (n = 1)	0.0	0.0	100.0
Spindle cell carcinomas (n = 1)	0.0	0.0	0.0
Tubular carcinoma (n = 5)	40.0	20.0	0.0
Tubulopapillary carcinoma (n = 24)	20.8	20.8	20.8

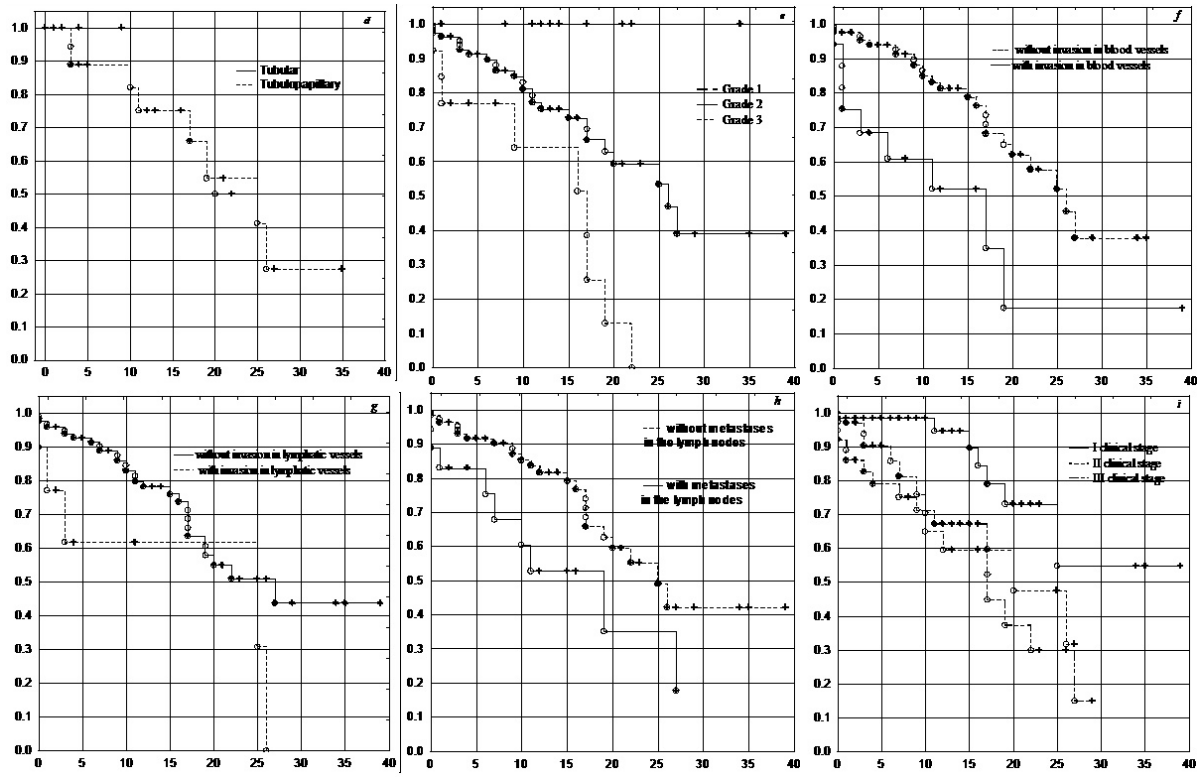

Fig. 1. Kaplan-Meier overall survival curves for bitches with tumours of the mammary gland after surgical treatment depending on *a*, *b*, *c*, *d* histological types; *e* – histological grade of malignancy; *f* – invasion in blood vessels; *g* – invasion in lymphatic vessels; *h* – damage to lymph nodes; *i* – clinical stages; + uncompleted monitoring, circles indicate complete monitoring – death or euthanasia due to progression of tumour process, the horizontal axis reflects life expectancy in months, vertical axis – share of survived individuals

Histological evaluation was significantly associated with the general life expectancy and median survival, it was higher in cases of malignancy grades I compared with cases of tumors of malignancy of grades II and III (P < 0.005, Table 2, Fig. 1e).

Table 2

Median survival and mean life expectancy at different histological degrees of malignancy of mammary tumours in 142 bitches

Grade	n	Median	x ± SD
I	13	13	12.8 ± 9.5
II	115	6	9.1 ± 9.2
III	13	5	9.4 ± 7.8

Intratumoral vascular invasion was observed in 17 cases included in the study, the animals with histological signs of invasion into blood vessels had statistically shorter median survival (6 months compared with 7 months, P = 0,0034, Fig. 1f). Invasion to the vessels was seen in 10 animals, they also had shorter median survival (2.5 against 7.5 months P = 0.0035, Fig. 1g). A total of 19 animals were observed to have metastatic lesions to lymph nodes, no significant difference in median survival was seen, but the average survival time was significantly higher than in bitches without involvement of lymph nodes (P = 0.007, Fig. 1h). No significant difference was seen in life expectancy of animals depending on size of tumour. Clinical stage according to Owen negatively affected the life expectancy (P = 0.015, Table 3, Fig. 1i).

Review analysis of ROC curve revealed that ROC discriminative capacity of vet-NPI and clinical stage according to Owen for identification of tumour-related death was high (area under the curve [AUC] 0.80; 95% CI – 0.72–0.88, P = 0.0012 for vet-NPI and AUC 0.68; 95% CI – 0.58–0.78, P = 0.0015 for clinical stage according to Owen, Fig. 2b). Pearson correlation coefficient between clinical stage and vet-NPI equaled 0.68 (P = 0,001). Thus, vet-NPI is a more accurate predictor of tumour-related death compared with the clinical stage. Median survival of animals with vet-NPI index lower than 4 was statistically higher (9 months) compared with the animals with the index higher than 4 (7 months, P = 0.00019, Fig. 2a). At the same time, the said difference gradually increased starting from the 10th month of the follow-up.

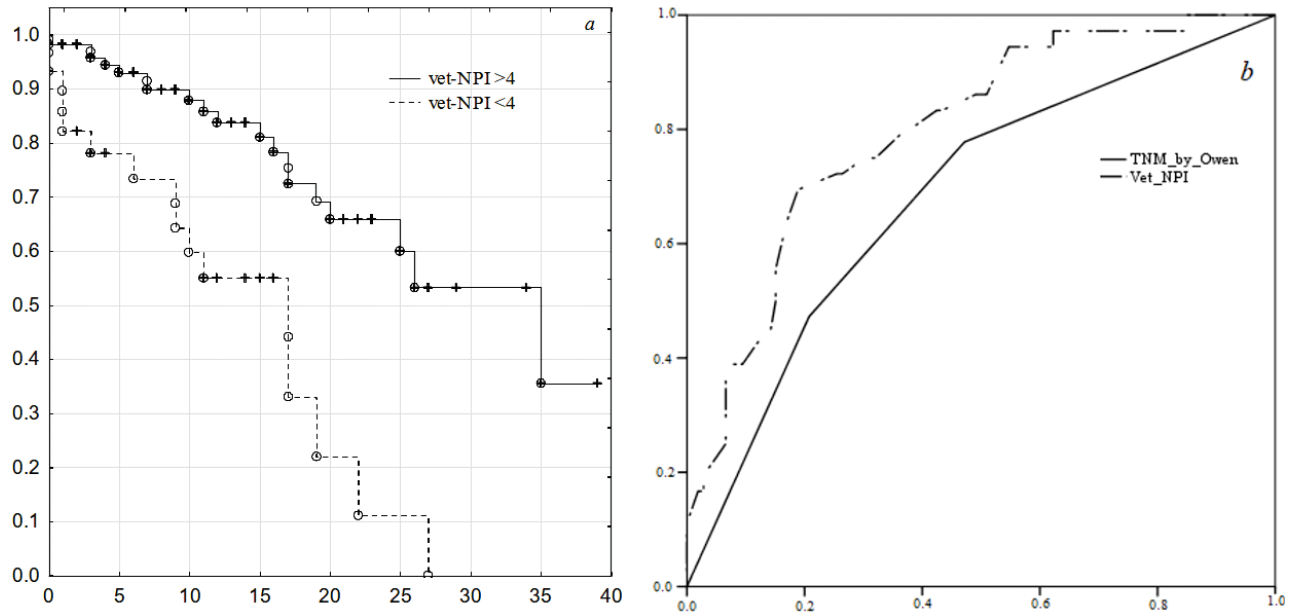

Fig. 2. Characteristics of bitches divided according to veterinary-adapted Nottingham prognostic index:
a – Kaplan-Meier overall survival curve of bitches with tumours of the mammary gland after surgical treatment depending on veterinary-adapted Nottingham prognostic index and clinical stage according to Owen (+ indicates uncompleted monitoring, circle indicates completed monitoring – death or euthanasia due to progression of tumour process, horizontal axis shows life expectancy in months, vertical axis – share of survived individuals); *b* – ROC-curve of predicting significance of veterinary-adapted Nottingham prognostic index and clinical stage according to Owen;
horizontal axis – 1-specificity, vertical axis – sensitivity

Table 3
Median survival and mean life expectancy
at various stages of mammary tumours in cohort of 142 bitches

Stage	n	median	x ± SD
I	63	9	10.0 ± 9.6
II	39	6	8.4 ± 8.0
III	39	8	9.3 ± 8.7

Discussion

Over the recent years, the prognoses in cases of mammary tumours have become more relevant. To choose optimum methods of diagnosing and treating tumour disease it is important to convert the characteristics of the tumour into prognostic data. Absence of efficient prognosis protocols is related to complexity of interpretation of the data due to application of different methods of studying tumour components (Matos et al., 2012). Taking into account that metastases and relapses develop in around 35–70% of bitches after surgical treatment (Collivignarelli et al., 2021), evaluating the prognostic markers is the key element to improve the therapeutic strategies for treating cancer patients (Boracchi et al., 2021).

The results presented in this paper correlate with our previous studies that indicate presence of signs of lymphatic / angioinvasion against the background of certain types of tumours of the mammary gland even at the first stages of the development and therefore significance of detecting them for prognosing the biological behaviour of the tumour (Kovalenko & Bilyi, 2021). The determined patterns of significance of presence of tumour emboli for evaluation of the survival rate of cancer patients confirm the limited possibilities of clinical classifications. In particular, grading according to Peña system has no effect on the prognosis during application to multiple primary tumours of the mammary gland in dogs (Pecile et al., 2021).

Histologic type of tumours can carry significance for the prognosis of mammary tumours in dogs. Dogs with benign tumours and carcinomas that emerge during benign mixed tumours have favourable prognosis in cases of complex carcinomas and simple tubular carcinomas, and also long survival. In cases of simple tubulopapillary carcinoma, interductal pappilary carcinoma, carcinoma and malignant myoepithelioma, the risk of tumour-related death increases 10-fold. Prognoses are even worse for adenosquamous carcinoma (MST = 18 months), comedocarcinoma (MST = 14 months) and solid carcinoma (MST = 8 months), anaplastic carcinoma (MST = 3 months) and carcinosarcoma (MST = 3 months), the last two histological types of which also have the highest indicators of metastasis (89% and 100% respectively) (Rasotto et al., 2017). Lymph invasion is associated with comedocarcinoma, anaplastic carcinoma and inflammatory carcinoma (Im et al., 2014).

Analysis of the status of the regional lymph nodes, histological type and vascular invasion during spontaneous non-inflammatory carcinomas of the mammary glands in bitches by determining the proliferation index, density of microvessels, vascular expression of growth factor receptor 2 (VEGFR2) using respectively anti-proliferating cell nuclear antigen (PCNA), von Willebrand factor and anti-Flk-1, revealed the correlation of survival of patients only with high degree of expression of VEGFR2 (Diessler et al., 2017). The obtained results are coherent with a number of articles the authors of which emphasized the confirmed fact of the prognostic significance of the lymphovascular invasion and invasion into lymph nodes for unfavourable course in cases of neoplasias of the mammary gland, whereas dependence of patients' survival on angioinvasion is studied insufficiently and therefore is debatable (Gujam et al., 2014).

Lymphatic and vascular invasions were seen to be significant for single-factor analysis; presence of vascular invasion is an independent prognostic factor, while lymphatic invasion cannot be used as a predictor of course of the disease in multi-dimensional analysis. The authors note possible development of remote metastases in patients with invasion of cancer cells to lymph nodes against the background of absence of angioinvasion (Fujii et al., 2014). Vascular invasion may be an independent factor for unfavourable prognosis, though no dependence between lymphovascular invasion and size of mammary tumour of bitches was determined (Muhammadnejad et al., 2012).

In veterinary medicine, the conditional survival of bitches after mastectomy is proposed to be determined using such significant parameters as lymphatic and vascular invasions, aside from age of patients, stage and histological type of cancer and clinical status. At the same time, research has revealed that long-term prognosis to a greater degree depends on the presence of tumour emboli in lymph and blood vessels (Chocteau et al., 2021). Lymphatic invasion in case of mammary tumours in bitches with developed metastases in regional lymph nodes and remote tissues and the organs is accompanied by significant increase in cancer antigen (CA 15.3) content in blood, which is one of the most significant cancer markers in

cases of this pathology (Campos et al., 2012). Despite the determined patterns, there is a need for further studies that would aim at determining pathogenic mechanisms of the development of tumour emboli in blood and lymph vessels and their effect on the course of the disease, which would be a basis for developing effective measures of prophylaxis and treatment of neoplasias of canine mammary glands.

Conclusions

Currently, behaviour of mammary tumours in bitches after mastectomy is the most significant factor for predicting the outcome. The most effective predictors that allow one to determine potential aggressiveness of tumour, life expectancy and relapse-free period in patients are neoplasia emboli in blood and lymph vessels, and also regional lymph nodes. The risk level of disease progression and tumour-related death of bitches correlates with clinical stage and histological type of neoplasias, intratumoral angio- and lymphatic invasions, but not with the size of the disease site. For the purpose of prognosing the course of oncological process in dogs suffering mammary tumours, it is practical to use vet-NPI. For the best understanding of pathogenic links of oncogenesis, there is a need for further studies of the mechanisms of migration of cancer cells out of the borders of the neoplasia tissues and their dissemination in the organism.

References

Amini, P., Nassiri, S., Malbon, A., & Markkanen, E. (2020). Differential stromal reprogramming in benign and malignant naturally occurring canine mammary tumours identifies disease-modulating stromal components. Scientific Reports, 10(1), 5506.

Badowska-Kozakiewicz, A. M., & Malicka, E. (2010). Expression of cyclooxygenase-2 in neoplasms of the mammary gland in bitches. Polish Journal of Veterinary Sciences, 13(2), 337–342.

Benavente, M. A., Bianchi, C. P., & Aba, M. A. (2019). Expression of oxytocin receptors in canine mammary tumours. Journal of Comparative Pathology, 170, 26–33.

Biondi, L. R., Tedardi, M. V., Gentile, L. B., Chamas, P., & Dagli, M. (2021). Quantification of global DNA methylation in canine mammary gland tumors via immunostaining of 5-methylcytosine: Histopathological and clinical correlations. Frontiers in Veterinary Science, 8, 628241.

Boracchi, P., Roccabianca, P., Avallone, G., & Marano, G. (2021). Kaplan-Meier curves, Cox model, and P-values are not enough for the prognostic evaluation of tumor markers: Statistical suggestions for a more comprehensive approach. Veterinary Pathology, 58(5), 795–808.

Borghesi, J., Giancoli Kato Cano da Silva, M., de Oliveira Pimenta Guimarães, K., Mario, L. C., de Almeida da Anunciação, A. R., Silveira Rabelo, A. C., Gonçalves Hayashi, R., Lima, M. F., Miglino, M. A., Oliveira Favaron, P., & Oliveira Carreira, A. C. (2021). Evaluation of immunohistopathological profile of tubular and solid canine mammary carcinomas. Research in Veterinary Science, 136, 119–126.

Brunetti, B., Bacci, B., Angeli, C., Benazzi, C., & Muscatello, L. V. (2021). p53, ER, and Ki67 expression in canine mammary carcinomas and correlation with pathological variables and prognosis. Veterinary Pathology, 58(2), 325–331.

Burrai, G. P., Gabrieli, A., Moccia, V., Zappulli, V., Porcellato, I., Brachelente, C., Pirino, S., Polinas, M., & Antuofermo, E. (2020). A statistical analysis of risk factors and biological behavior in canine mammary tumors: A multicenter study. Animals, 10(9), 1687.

Campos, L. C., Lavalle, G. E., Estrela-Lima, A., Melgaço de Faria, J. C., Guimarães, J. E., Dutra, Á. P., Ferreira, E., de Sousa, L. P., Rabelo, É. M., Vieira da Costa, A. F., & Cassali, G. D. (2012). CA15.3, CEA and LDH in dogs with malignant mammary tumors. Journal of Veterinary Internal Medicine, 26(6), 1383–1388.

Cassali, G. D., Bertagnolli, C. A., Ferreira, E., Damasceno, K. A., Gamba, C. O., & de Campos, C. B. (2012). Canine mammary mixed tumours: A review. Veterinary Medicine International, 2012, 274608.

Chocteau, F., Mordelet, V., Dagher, E., Loussouarn, D., Abadie, J., & Nguyen, F. (2021). One-year conditional survival of dogs and cats with invasive mammary carcinomas: A concept inspired from human breast cancer. Veterinary and Comparative Oncology, 19(1), 140–151.

Collivignarelli, F., Tamburro, R., Aste, G., Falerno, I., Del Signore, F., Simeoni, F., Patsikas, M., Gianfelici, J., Terragni, R., Attorri, V., Carluccio, A., & Vignoli, M. (2021). Lymphatic drainage mapping with indirect lymphography for canine mammary tumors. Animals, 11(4), 1115.

Costa-Santos, K., Damasceno, K., Portela, R. D., Santos, F. L., Araújo, G. C., Martins-Filho, E. F., Silva, L. P., Barral, T. D., Santos, S. A., & Estrela-Lima, A. (2019). Lipid and metabolic profiles in female dogs with mammary carcinoma receiving dietary fish oil supplementation. BMC Veterinary Research, 15(1), 401.

Diessler, M. E., Castellano, M. C., Portiansky, E. L., Burns, S., & Idiart, J. R. (2017). Canine mammary carcinomas: Influence of histological grade, vascular invasion, proliferation, microvessel density and VEGFR2 expression on lymph node status and survival time. Veterinary and Comparative Oncology, 15(2), 450–461.

Elston, C. W., & Ellis, I. O. (1991). Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: Experience from a large study with long-term follow-up. Histopathology, 19(5), 403–410.

Fujii, T., Yajima, R., Hirakata, T., Miyamoto, T., Fujisawa, T., Tsutsumi, S., Ynagita, Y., Iijima, M., & Kuwano, H. (2014). Impact of the prognostic value of vascular invasion, but not lymphatic invasion, of the primary tumor in patients with breast cancer. Anticancer Research, 34(3), 1255–1259.

Goldschmidt, M., Peña, L., Rasotto, R., & Zappulli, V. (2011). Classification and grading of canine mammary tumors. Veterinary Pathology, 48(1), 117–131.

Guil-Luna, S., Stenvang, J., Brünner, N., De Andrés, F. J., Rollón, E., Domingo, V., Sánchez-Céspedes, R., Millán, Y., & Martín de Las Mulas, J. (2014). Progesterone receptor isoform A may regulate the effects of neoadjuvant aglepristone in canine mammary carcinoma. BMC Veterinary Research, 10, 296.

Gujam, F. J., Going, J. J., Edwards, J., Mohammed, Z. M., & McMillan, D. C. (2014). The role of lymphatic and blood vessel invasion in predicting survival and methods of detection in patients with primary operable breast cancer. Critical Reviews in Oncology/Hematology, 89(2), 231–241.

Haybittle, J. L., Blamey, R. W., Elston, C. W., Johnson, J., Doyle, P. J., Campbell, F. C., Nicholson, R. I., & Griffiths, K. (1982). A prognostic index in primary breast cancer. British Journal of Cancer, 45(3), 361–366.

Im, K. S., Kim, N. H., Lim, H. Y., Kim, H. W., Shin, J. I., & Sur, J. H. (2014). Analysis of a new histological and molecular-based classification of canine mammary neoplasia. Veterinary Pathology, 51(3), 549–559.

Kovalenko, M., & Bilyi, D. (2021). Prognostic value of vascular invasion in breast tumours in she-dogs (pilot study). Scientific Horizons, 24(2), 54–61.

Levi, M., Muscatello, L. V., Brunetti, B., Benazzi, C., Parenti, F., Gobbo, F., Avallone, G., Bacci, B., Zambon, E., Valenti, P., & Sarli, G. (2021). High intrinsic expression of P-glycoprotein and breast cancer resistance protein in canine mammary carcinomas regardless of immunophenotype and outcome. Animals, 11(3), 658.

Levi, M., Peña, L., Alonso-Díez, A., Brunetti, B., Muscatello, L. V., Benazzi, C., Pérez-Alenza, M. D., & Sarli, G. (2019). P-glycoprotein and breast cancer resistance protein in canine inflammatory and noninflammatory grade III mammary carcinomas. Veterinary Pathology, 56(6), 840–847.

Matos, A. J., Baptista, C. S., Gärtner, M. F., & Rutteman, G. R. (2012). Prognostic studies of canine and feline mammary tumours: The need for standardized procedures. Veterinary Journal, 193(1), 24–31.

Muhammadnejad, A., Keyhani, E., Mortazavi, P., Behjati, F., & Haghdoost, I. S. (2012). Overexpression of Her-2/neu in malignant mammary tumors; translation of clinicopathological features from dog to human. Asian Pacific Journal of Cancer Prevention, 13(12), 6415–6421.

Mysak, A., Kielbowicz, Z., Khomyn, N., Pritsak, V., & Gutyj, B. (2018). Graphically x-ray and ultrasound diagnostics for monitnoring neoplasia of the mammary gland in bitches. Ukrainian Journal of Ecology, 8(1), 386–393.

Nicchio, B. O., Barrouin-Melo, S. M., Machado, M. C., Vieira-Filho, C. H., Santos, F. L., Martins-Filho, E. F., Barbosa, V. F., Barral, T. D., Portela, R. W., Damasceno, K. A., & Estrela-Lima, A. (2020). Hyperresistinemia in obese female dogs with mammary carcinoma in benign-mixed tumors and its correlation with tumor aggressiveness and survival. Frontiers in Veterinary Science, 7, 509.

Okada, S., Furuya, M., Fukui-Kaneshige, A., Nakanishi, H., Tani, H., & Sasai, K. (2021). HSP110 expression in canine mammary gland tumor and its correlation with histopathological classification and grade. Veterinary Immunology and Immunopathology, 232, 110171.

Pecile, A., Groppetti, D., Ferrari, R., Grieco, V., Giudice, C., Spediacci, C., Stefanello, D., & Boracchi, P. (2021). Solitary and multiple simultaneous malignant epithelial mammary tumours in dogs: An explorative retrospective study. Research in Veterinary Science, 135, 153–161.

Peña, L., Andrés, P. J. D., Clemente, M., Cuesta, P., & Pérez-Alenza, M. D. (2013). Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: Relationship with clinical and histological characteristics. Veterinary Pathology, 50(1), 94–105.

Pöschel, A., Beebe, E., Kunz, L., Amini, P., Guscetti, F., Malbon, A., & Markkanen, E. (2021). Identification of disease-promoting stromal components by comparative proteomic and transcriptomic profiling of canine mammary tumors using laser-capture microdissected FFPE tissue. Neoplasia, 23(4), 400–412.

Rasotto, R., Berlato, D., Goldschmidt, M. H., & Zappulli, V. (2017). Prognostic significance of canine mammary tumor histologic subtypes: An observational cohort study of 229 cases. Veterinary Pathology, 54(4), 571–578.

Ressel, L., Puleio, R., Loria, G. R., Vannozzi, I., Millanta, F., Caracappa, S., & Poli, A. (2013). HER-2 expression in canine morphologically normal, hyperplastic and neoplastic mammary tissues and its correlation with the clinical outcome. Research in Veterinary Science, 94(2), 299–305.

Santos, M., Correia-Gomes, C., Marcos, R., Santos, A., De Matos, A., Lopes, C., & Dias-Pereira, P. (2015). Value of the Nottingham histological grading parame-

ters and Nottingham prognostic index in canine mammary carcinoma. Anticancer Research, 35(7), 4219–4227.
Terajima, M., Taga, Y., Brisson, B. K., Durham, A. C., Sato, K., Uzawa, K., Saito, T., Hattori, S., Sørenmo, K. U., Yamauchi, M., & Volk, S. W. (2021). Collagen molecular phenotypic switch between non-neoplastic and neoplastic canine mammary tissues. Scientific Reports, 11(1), 8659.
Terzaghi, L., Banco, B., Groppetti, D., Dall'Acqua, P. C., Giudice, C., Pecile, A., Grieco, V., Lodde, V., & Luciano, A. M. (2020). Progesterone receptor membrane component 1 (PGRMC1) expression in canine mammary tumors: A preliminary study. Research in Veterinary Science, 132, 101–107.
Tesi, M., Millanta, F., Poli, A., Mazzetti, G., Pasquini, A., Panzani, D., Rota, A., & Vannozzi, I. (2020). Role of body condition score and adiponectin expression in the progression of canine mammary carcinomas. Veterinary Medicine and Science, 6(3), 265–271.
Torres, C. G., Iturriaga, M. P., & Cruz, P. (2021). Hormonal carcinogenesis in canine mammary cancer: Molecular mechanisms of estradiol involved in malignant progression. Animals, 11(3), 608.
Tuna, O., Baykal, A., Sozen Kucukkara, E., Ozten, O., Deveci Ozkan, A., Guney Eskiler, G., Kamanli, A. F., Bilir, C., Yildiz, S. Z., Kaleli, S., Ucmak, M., Kasikci, G., & Lim, H. S. (2021). Efficacy of 5-aminolevulinic acid-based photodynamic therapy in different subtypes of canine mammary gland cancer cells. Lasers in Medical Science, 2021, in press.
Yamagami, T., Kobayashi, T., Takahashi, K., & Sugiyama, M. (1996). Prognosis for canine malignant mammary tumors based on TNM and histologic classification. The Journal of Veterinary Medical Science, 58(11), 1079–1083.