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Clinical presentation, histological findings, and prognosis in female dogs with mixed mammary neoplasms

[*Apresentação clínica, achados histológicos e prognóstico em cadelas com neoplasias mamárias mistas*]

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ABSTRACT

The aim of this study was to describe the clinical and morphological characteristics of mixed mammary neoplasms and verify what characteristics affect the prognosis of female dogs with carcinomas in mixed tumors and carcinosarcomas. This was a retrospective study of 67 female dogs that underwent mastectomies and were diagnosed with benign mixed tumors (n=13), carcinomas in mixed tumors (n=44) and carcinosarcomas (n=10). Data regarding the clinical and histological aspects of the neoplasms were collected and the relation with specific survival times, and hazard ratios (HR) in 24 months was calculated. In univariate analysis, the diagnosis of carcinosarcoma (HR 8.26, p=0.006), carcinomatous areas with micropapillary or solid patterns (HR 17.49; p=0. 001) and lymph node metastasis (HR 7.07;p=0.020) were associated with specific survival. In multivariable analysis, only micropapillary or solid pattern (HR=16.34; p=0.007) remained independent factor associated with lower specific survival. Micropapillary or solid carcinomatous patterns were associated with shorter specific survival time (p=0.002) among animals with carcinomas in mixed tumors. Among the carcinosarcomas, lymph node metastasis (p=0.010) was associated with a shorter specific survival time. In conclusion, carcinomas in mixed tumors and carcinosarcomas vary in prognosis depending on the carcinomatous proliferation patterns and spread of the disease.

Keywords: canine, mammary, mixed, tumor

RESUMO

O objetivo deste estudo foi descrever as características clínicas e morfológicas das neoplasias mamárias mistas e verificar quais características interferem no prognóstico de cadelas com carcinomas em tumores mistos e carcinossarcomas. Este foi um estudo retrospectivo de 67 cadelas, que foram submetidas a mastectomias e diagnosticadas com tumores mistos benignos (n = 13), carcinomas em tumores mistos (n = 44) e carcinossarcomas (n = 10). Foram coletados dados sobre os aspectos clínicos e histológicos das neoplasias e a relação com o tempo de sobrevida específica e a razão de risco (OR) em 24 meses foram calculadas. Na análise univariada, o diagnóstico de carcinossarcoma (HR 8,26; p=0,006), as áreas carcinomatosas com padrão micropapilar e sólido (HR 17,49; p= 0,001) e metástase linfonodal (HR 7,07; p=0,020) foram associadas à menor sobrevida específica. Na análise multivariável, apenas o padrão micropapilar ou sólido (HR=16,34; p=0,007) permaneceu como fator independente associado à sobrevida. Proliferações carcinomatosas micropapilares ou sólidas (p = 0,002) foram associadas a tempos de sobrevida específica mais curtos entre os animais com carcinomas em tumor misto. Entre os pacientes com carcinossarcoma, metástases em linfonodos (p = 0,010) foram associadas a um menor tempo de sobrevida específica. Em conclusão, os resultados mostraram que os carcinomas em tumores mistos e os carcinossarcomas podem ter prognóstico variável, dependendo do padrão de proliferação carcinomatosa e da disseminação linfática da doença.

Palavras-chave: canino, mamário, misto, tumor

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INTRODUCTION

Mixed mammary neoplasms are relatively common in female dogs (Toríbio *et al*., 2012; Salas *et al*., 2015; Nunes *et al*., 2019). Among these neoplasms, carcinomas in mixed tumors account for a high proportion of neoplasms diagnosed in female dogs. Carcinomas in mixed tumors are constituted by epithelial (malignant and benign) and myoepithelial proliferation with foci of mesenchymal cell producing cartilage and/or bone (Misdorp *et al*., 1999; Cassali *et al*., 2014). Epithelial proliferations may present diverse histological patterns within the tumor mass, such as tubular, papillary, micropapillary, and solid patterns. This histological complexity can also be observed in carcinosarcomas. Carcinosarcomas are less common than carcinomas in mixed tumors, and exhibit combinations of carcinomatous and sarcomatous components (Misdorp *et al*., 1999; Cassali *et al*., 2017).

In general, the prognosis for animals with malignant mammary neoplasms may vary significantly, depending on the clinical and histological characteristics of the neoplasm, including the clinical stage, histological type, intratumorally angiolymphatic invasion, and histological grade, among other factors (Rasoto *et al*., 2017, Chocteau *et al*., 2019 , Canadas *et al*., 2019; Cassali *et al*., 2020).

Immunohistochemical markers such as estrogen and progesterone receptors, MIB-1, cyclooxygenase-2 (COX-2), epidermal growth factor receptors (HER2 and EGFR), and cytokeratins have also been associated with prognosis in female dogs with mammary neoplasms (Gama *et al*., 2008; Sassi *et al*., 2010; Ribeiro *et al*., 2012; Abadie *et al*., 2017). Despite demonstrating usefulness and promise, immunohistochemistry is still not a routine tool in veterinary medicine in some countries, due to the cost and limited laboratory diagnostic services.

Female dogs that have been diagnosed with carcinomas in mixed tumor generally have a better prognosis than animals with other types of carcinomas (Yamagami *et al*., 1996; Rasotto *et al*., 2017). Carcinosarcomas have been generally associated with shorter survival times and higher rates of distant metastasis (Rasotto *et al*., 2017; Nunes *et al*., 2019). However, little is known about the influence of other clinical and histological aspects on the prognosis of these types of neoplasms (Cassali *et al*., 2017).

The purpose of this study is to describe the clinical and morphological characteristics of carcinomas in mixed tumors and carcinosarcomas in female dogs and verify the characteristics that affect prognosis.

MATERIAL AND METHODS

This retrospective study included female dogs that underwent the surgical excision of mammary tumors at a university veterinary hospital (Hospital de Clínicas Veterinárias, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul) from March 2014 to March 2016. The surgical specimens (mammary tumors and lymph nodes) were obtained from archives at the veterinary pathology unit Setor de Patologia Veterinária at Universidade Federal do Rio Grande do Sul. The inclusion criteria were diagnoses of benign mixed tumor (BMT), carcinoma in mixed tumor (CMT), or carcinosarcoma (CS). Cases involving previous treatment for mammary tumors or chemotherapy, coexistent mammary neoplasms of other histological types, and malignant neoplasms in other types of tissue or systems were excluded.

This study was approved by the University Animal Ethics Committee (Comitê de Ética em Uso de Animais da Universidade Federal do Rio Grande do Sul), and registered under approval number 28805, as well as by the veterinary research institute Instituto de Pesquisas Veterínarias Desidério Finamor and registered under approval number 16/14. Including the animals in our study did not interfere with their surgical indications, chemotherapy, or any other form of recommended treatment for these patients.

Patient medical records and exam results (radiographic and ultrasonographic) were analyzed to collect information regarding age, tumor location, tumor distribution, tumor size (measured in their largest diameter), abnormal radiologic or ultrasonographic findings suggesting distant metastasis, metastasis to primary tumor-draining lymph nodes, and outcome. Telephone interviews with owners were also conducted every six months, over 24

months. During these conversations, the owners were questioned about the health of their pets, and whether they had noticed any new nodules around the surgical scar or the preserved contralateral mammary chain. They were also asked about other clinical signs that could indicate metastasis. If deaths occurred, they were asked to report the date and situation or cause of death.

In all the female dogs included in study, a pre - surgical assessment for distant metastasis was carried out through a radiographic examination of the thorax (preferably with three views: the right lateral view, the left lateral view, and the anterior-posterior view) and abdominal ultrasonography. The tumor-draining lymph nodes were removed surgically (n=40) and examined to detect metastasis through histopathology and immunohistochemistry.

All cases with available lymph nodes were clinically staged based on tumor diameter, radiology and ultrasound exams and lymph node histopathology results, according to the parameters established in the Tumor-Node-Metastasis (TNM) system (Owen, 1980; Sorenmo *et al.*, 2013).

The histological revision was performed using histological sections stained with haematoxylin and eosin (Zappulli *et al.*, 2019; Cassali *et al.*, 2014). Neoplasms were classified as BMTs if they presented both epithelial (ductal) and myoepithelial proliferation with foci of mesenchymal cells producing an identifiable product, usually cartilage and/or bone, and variable amounts of fibrous stroma. The epithelial component consists of non-infiltrating cubic or columnar epithelial cells with discrete anisokaryosis and anisocytosis, and rare or absent mitosis. BMTs presenting malignant epithelial proliferation were classified as CMTs. In particular, the diagnosis of carcinoma was based on the presence of atypical epithelial cells with marked anisokaryosis and anisocytosis, pluristratification, frequent mitosis and stromal invasion. Neoplasms were classified as CS if they presented carcinomatous and sarcomatous cell proliferation foci (chondrosarcoma, osteosarcoma, or fibrosarcoma). Characteristics such as accentuated anisocytosis and anisokaryosis, evident nucleoli, binucleated or multinucleated cells, and frequent mitosis were decisive for a sarcoma component classification.

Vascular invasion and intratumor necrosis were other histological parameters that we assessed in malignant neoplasms. In the cases where the tumor-draining lymph node was referred for histopathology, the histological sections were revised. The presence of neoplastic cells in the lymphatic parenchyma or capsular sinuses led to a classification of lymph node metastasis.

Histological sections 4µm thick were obtained from each lymph node and collected on histologically polarized slides for immunohistochemistry (IHC) analysis to confirm lymph node status (metastatic deposits and the nature of metastatic cells). The lymph nodes from carcinomas in mixed tumors and carcinosarcoma cases were re-evaluated through IHC using antibodies against human cytokeratins (CK) AE1/AE3 (Novocastra ^{TM} Liquid Mouse Monoclonal Anti-Multi-Cytokeratin, Product code NCL-L-AE1/AE3, Leica Biosystems; Newcastle, United Kingdom;1: 150) to detect epithelial metastasis. Vimentin expression (Novocastra ^{TM} Liquid Mouse Monoclonal Antibody Vimentin, Product code NCL-L - VIM-V9; Leica Biosystems, Newcastle, United Kingdom; 1: 800) was analyzed in the regional lymph node samples from the female dogs with carcinosarcoma to detect mesenchymal metastasis. The slides were deparaffinized in xylene and rehydrated in a series of progressively diluted alcohols. For antigenic recovery, the sections were incubated in citrate buffer (Recovery solution, Dako Cytomation, Carpinteria, CA, USA) pH: 6.0, in a water bath for 20 minutes.

To block endogenous peroxidase, the slides were incubated in 0.3% hydrogen peroxide and absolute methanol for 15 minutes at room temperature. Subsequently, the incubation with the primary antibody was performed for one hour followed by incubation in the Novolink Polymer Detection System (Leica Biosystems, Newcastle upon Tyne, United Kingdom - Leika), according to the manufacturer's instructions. Finally, the sections were exposed to chromogen 3.3 - diaminobenzidine 4 HCL (DAB) (DAB Substrate System, Dako, Carpinteria, CA, USA), and the slides were counterstained with Harris haematoxylin.

Sections of canine mammary tumors with confirmed positivity for vimentin and cytokeratin AE1 / AE3 were used as positive controls. The negative control was obtained by omitting the primary antibody.

The student's t-test for independent samples was used to compare the mean tumors sizes and ages among histological types. For the other analyses, the clinical and histological variables were stratified as follows: age (<10 years and ≥ 10 years), tumor location (thoracic mammary glands, abdominal mammary glands, inguinal glands, or multiple sites), tumor distribution (single or multiple), tumor size (≤5.0 cm and> 5.0 cm), necrosis (absent or present), pattern of the malignant epithelial component (tubular / papillary or micropapillary / solid), vascular invasion (absent or present), lymph node metastasis (absent or present) and clinical stage (I, II or III and IV or V).

Two-tailed Pearson chi-square tests or Fisher's exact tests (if more than 25% of the cells have an expected value of less than 5) were used to evaluate the association between clinical and histological variables and histological types (BMT vs CMT, and CMT vs CS).

The same tests were used to assess possible associations between the type of carcinomatous pattern in the carcinoma in mixed tumor, and clinical and morphological variables.

Specific survival was defined as the time in days between the date of the surgical excision of the mammary tumor and the date of death of the animal following deterioration of health, radiological images of distant metastasis, or euthanasia.

All animals that were alive on the last date in their medical records were censored, as well as those that died from causes unrelated to cancer or undetermined causes.

The Kaplan-Meier estimate was used to calculate specific survival functions. Survival curves were created for histologic types, age, tumor location, tumor numbers, tumor size, necrosis, carcinomatous pattern, vascular invasion, lymph node metastasis and clinical stage. Separate survival curves, stratified by clinical and histological variables, were created for female dogs with carcinomas in mixed tumors and carcinosarcomas. The Log-rank test was used to compare the survival functions for each variable. Values were considered statistically significant when $p < 0.05$. None of the animals with benign mixed tumors died. For this reason, they were not included in survival analysis.

To identify the independent effect of the clinical and histological variables on the hazard ratio (HR) for death in female dogs from the malignant tumor group, a Cox proportional hazards regression model was used.

Only those variables with statistically significant crude (unadjusted) HR ($p<0.05$) were included in the adjustment in the multivariable analysis. To adjust the model, a forward selection procedure was used to select variables, with a cut-off p value of 0.05.

Statistical analysis was performed using STATA software (version 12.0, Stata Corporation, College Station, TX).

RESULTS

During the study, 289 female dogs with mammary tumors were given care at the university veterinary hospital. Among these animals, 156 (53.97%), were diagnosed with some type of matrix producing neoplasm, and 67 of these were chosen for our study. Thirteen were diagnosed with BMT, 44 with CMT, and 10 with CS. The remaining patients presented factors for exclusion and were not considered in the analyses.

The clinical and histological characteristics of the tumors are presented in Table 1, according to the histological types. The average age of the female dogs with BMT was 9.2 years (ranging from 5 to 13 years), while the patients with CMT had an average age of 10.3 years (ranging from 2 to 16 years), and those with CS had an average age of 9.8 years (ranging from 3 to 12 years).

Table 1. Clinical and histological characteristics of mammary neoplasms with mesenchymal components (n=67)

Variables	TMB	CMT		CSS		
	n (/%)	n (/%)	p* value vs. TMB*	n (/%)	p* value vs. TMB*	p* value of CMT vs. CSS*
Age						
<10 years old	6 (46.1)	15(34.9)	0.462	2(20.0)	0.195	0.364
≥10 years old	7(53.8)	28(65.1)		8(80.0)		
Tumor location						
Cranial glands	4 (33.3)	12(27.9)	0.714	1(11.1)	0.258	0.290
Abdominal glands	7 (58.3)	13(30.2)	0.074	6(66.7)	0.528	0.039
Inguinal glands	0	11(25.6)	0.051	2 (2.2)	0.171	0.832
Multiple glands	1(8.3)	7(16.3)	0.490	0	0.571	0.193
Tumor numbers						
Simple	10 (76.9)	26(59.1)	0.278	8(80.0)	0.633	0.216
Multiple	3 (23.0)	18(40.1)		2(20.0)		
Tumor size						
≤5 cm	12(100.0)	24(54.5)	0.004	2(20.0)	p<0.0001	0.044
>5 cm	0	20(45.4)		8(80.0)		
Necrosis						
Negative	13(100.0)	17(38.6)	p<0.0001	3(30.0)	p<0.0001	0.610
Positive	0	27(61.4)		7(70.0)		
Carcinomatous pattern						
Papillary or tubular	-	40(90.9)	-	6(60.0)	-	0.013
Micropapillary or solid	-	4(9.09)		4(40.0)		
Vascular invasion						
Negative	13(100.0)	36(81.8)	0.097	6(66.7)	0.055	0.307
Positive	0	8 (18.2)		3(33.3)		
Lymph node-metastasis						
Negative	14(100.0)	27(84.4)	0.165	5(62.5)	0.042	0.137
Positive	0	5 (15.6)		3(37.5)		
Clinical stage						
I, II, and III	-	24(75.0)	-	4(50.0)	-	0.168
IV and V	-	8 (25.0)		4(50.0)		

Abbreviations: TMB= benign mixed tumor; CTM= carcinoma in mixed tumor; CS= carcinosarcoma
*The Pearson's chi-square test or Fisher's exact tests with 2-tailed analysis were used as appropriate.

The average of the maximum diameters among BMT was 0.9 cm (0.3-2.0 cm), 4.8 cm (0.2-5.0 cm) among CMT and 8.3 cm (2-15 cm) among CS. The average size of the BMT was significantly smaller than the average size of CMT (p<0.0001) and CS (p<0.0001). The CMT were of a smaller size than CS (p=0.003).

All benign mixed tumors were smaller than 3.0 cm. Among the CMT, 38.6% (n=17) were smaller than 3.0cm, 15.9% (n=7) measured between 3.0-5.0 cm and 45.4% (n=20) were larger than 5.0cm. Among the CS, 10% (n=1) were smaller than 3.0cm, 10% (n=1) measured between 3.0-5.0cm and 80.0% (n=8) were larger than 5.0cm. The portion of tumors larger than 5.0cm was bigger among the CS than the CMT (p=0.048).

Excision of the lymph nodes was performed in 32 female dogs with CTM and 8 with CSS. Histopathological and immunohistochemical evaluation of these nodes revealed epithelial metastasis in 20.0% (8/40).

Among the female dogs with CMT, 28.1 % 9/32 were classified as stage I, 15.6% (5/32) stage II, and 40.6% (n=13) stage III. During follow-up care, three of the patients presented radiology imaging suggestive of pulmonary metastasis and were re-evaluated as stage V cases. Among the female dogs with CS, 50.0% (5/10) were stage

III patients and 37.5% (3/10) were classified as stage IV. One presented radiology imaging suggestive of pulmonary metastasis during follow-up and was re-evaluated as a stage V patient. There was no significant difference in the distribution between earlier stages (I, II or III) and IV or V stages among female dogs with CMT and CS.

The mesenchymal proliferation in BMT was of the chondroid type in 84.6% (11/13) of the cases, and osteoid in 15.4% (2/13) of the cases. Among the CMT, 81.8% (36/44) presented chondroid mesenchymal proliferation, 6.8% (3/44) osteoid proliferation, and 11.3% (5/44) a chondroid/osteoid mix, besides the proliferation of myoepithelial cells. The CS presented mostly mixed sarcomatous proliferations (90%) and there was one case (10%) of osteosarcomatous proliferation.

There was no statistical difference between CMT and CS regarding age, tumor location, tumor numbers, necrosis, vascular invasion, or lymph node metastasis.

Among the CMT, 90.9% (40/44) presented carcinomatous proliferation with tubular or papillary patterns and 9.1% (4/44) showed areas with micropapillary or solid proliferations. Multiple gland involvement was less frequent among cases with tubular or papillary areas (p=0.06). There was no significant association between the different types of carcinomatous patterns and other variables tested (Table 2).

Table 2. Clinical and histological characteristics of the growth patterns of the malignant epithelial component in carcinomas in mixed tumors (n=44)

Variables	Tubular or papillary patterns n(%)	Micropapillary or solid patterns n (%)	p value *
<10 years old	15 (38.5)	0	0.166
≥10 years old	24 (61.5)	4 (100.0)	
Tumor location			
Cranial glands	12 (30.0)	0	0.364
Abdominal glands	13(32.5)	0	0.329
Inguinal glands	11(27.5)	0	0.402
Multiple glands	4(10.0)	3 (100.0)	0.003
Tumor Numbers			
Simple	24(60.0)	2(50.0)	0.545
Multiple	16(40.0)	2 (50.0)	
Tumor size			
≤5 cm	22(55.0)	2(50.0)	0.624
>5 cm	18(45.0)	2(50.0)	
Intratumoural necrosis			
Negative	17 (42.5)	0(0)	0.129
Positive	23(57.5)	4(100.0)	
Vascular invasion			
Negative	34(85.0)	2 (50.0)	0.145
Positive	6 (15.0)	2 (50.0)	
Lymph node metastasis			
Negative	25 (89.3)	2(50.0)	0.105
Positive	3 (10.7)	2(50.0)	
Clinical stage			
I, II and III	22 (78.6)	2 (50.0)	0.254
IV and V	6(21.4)	2 (50.0)	

Abbreviations: TMB= benign mixed tumor; CTM= carcinoma in mixed tumor; CS= carcinosarcoma
*The Pearson's chi-square test or Fisher's exact tests with 2-tailed analysis were used as appropriate.

Results of median/mean, 2 year-survival rate and Log-rank test, in relation to specific survival, are listed in Table 3. Specific survival curves were different between female dogs with carcinosarcomas and those with carcinomas in mixed tumors. Female dogs with CS showed

shorter specific survival than animals with carcinomas in mixed tumors (Log- rank test; p=0.001).

Other characteristics such as lymph node metastasis (Log-rank test, p= 0.007), vascular invasion (Log–rank test; p=0.042), carcinomatous areas with micropapillary or solid patterns (Log–rank test, p<0.0001), and stages IV-V (Log-rank test, p=0.046) were associated with shorter survival times among female dogs with malignant neoplasms.

Table 3. Median and mean survival times, survival rate and univariable Cox regressions for the association between the clinical and histological characteristics and specific survival in 54 female dogs with malignant mixed mammary tumors

Variables	Mean survival/ Median survival	2 year-survival rate (%)	Log –rank test	Univariate Cox Regression			Multivariate Cox Regression		
				HR	95% CI	P value	HR	95% CI	p value
Histological type									
CTM	716/ NR	41/44 (93.2)	0.001	Reference					
CSS	462/ NR	6/10 (60.0)		8.26	1.83-37.22	0.006			NS
Carcinomatouspattern									
Papillary or tubular	707/NR	43/46 (93.5)	p<0.0001	Reference			Reference		
Micropapillary or solid	426/571	4/8 (50.0)		17.49	3.11-98.33	0.001	16.34	2.1-124.89	0.007
Vascular invasion									
Negative	691/NR	38/42 (90.5)	0.042	Reference					
Positive	567/NR	8/11 (72.7)		4.32	0.93-20.07	0.061	-		-
Lymph node metastasi									
Negative	712/NR	28/31 (90.3)	0.007	Reference					NS
Positive	479/NR	5/8 (62.5)		7.07	1.36-36.54	0.020			
Clinical stage									
I, II, and III	696/NR	25/28 (89.3)	0.046	Reference					NS
IV and V	532/NR	8/12 (66.7)		4.09	0.9918.41	0.066			

Abbreviations: ms=mean survival time; MS=median survival time; HR= hazard ratio; CI, confidence interval.

The specific survival curves stratified by age, tumor location, tumor numbers, tumor size and necrosis, showed no statistical differences in the Log-rank test.

The univariable Cox regressions showed that the risk for death was highest for female dogs with carcinosarcoma than those with carcinoma in mixed tumors (HR 8.26, p=0.006). Neoplasms with carcinomatous areas with micropapillary or solid patterns (HR 17.49; p=0. 0.001) resulted in a higher risk of death than neoplasms with papillary or tubular areas. Female dogs with lymph node metastasis (HR 7.07, p=0.020) had a higher risk of death than animals with no lymph node metastasis.

The multivariable Cox regression model showed that a micropapillary or solid pattern (HR=16,34, p=0.007) remained independent factors associated with the risk of death among the female dogs.

Among the female dogs with carcinoma in mixed tumor, areas of micropapillary or solid carcinomatous proliferation demonstrated lower specific survival (Log-rank test, p=0.002) than those with tubular or papillary areas (Fig. 1). Specific survival curves of female dogs with carcinoma in mixed tumor, stratified by age, tumor location, tumor numbers, tumor size, necrosis, vascular invasion, lymph node metastasis and clinical stage, showed no statistical differences in the Log-rank test.

Among the female dogs with CS, lymph node metastasis resulted in a shorter survival time (Log-rank test, p=0.010) (Fig. 2) than female dogs with CS but no lymph node metastasis.

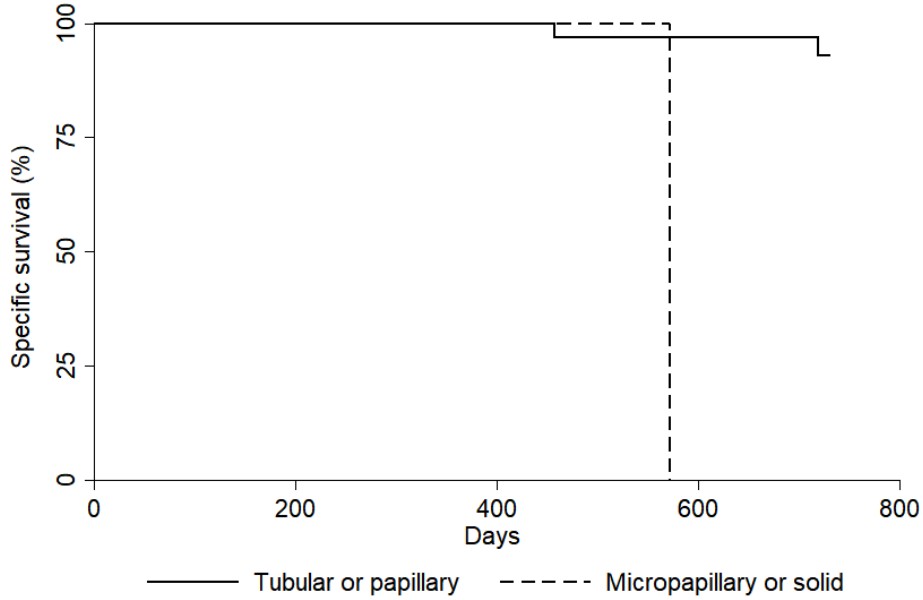

Figure 1. Kaplan-Meier specific survival curves of female dogs with carcinomas in mixed tumors (n=44). **A -** Female dogs with carcinomas in mixed tumors stratified by the growth pattern of the malignant epithelial component. Female dogs with neoplasms exhibiting micropapillary or solid areas (median of 571 days) demonstrated a lower probability of survival than animals with papillary or tubular proliferation (mean of 721 days); log-rank, p=0.002).

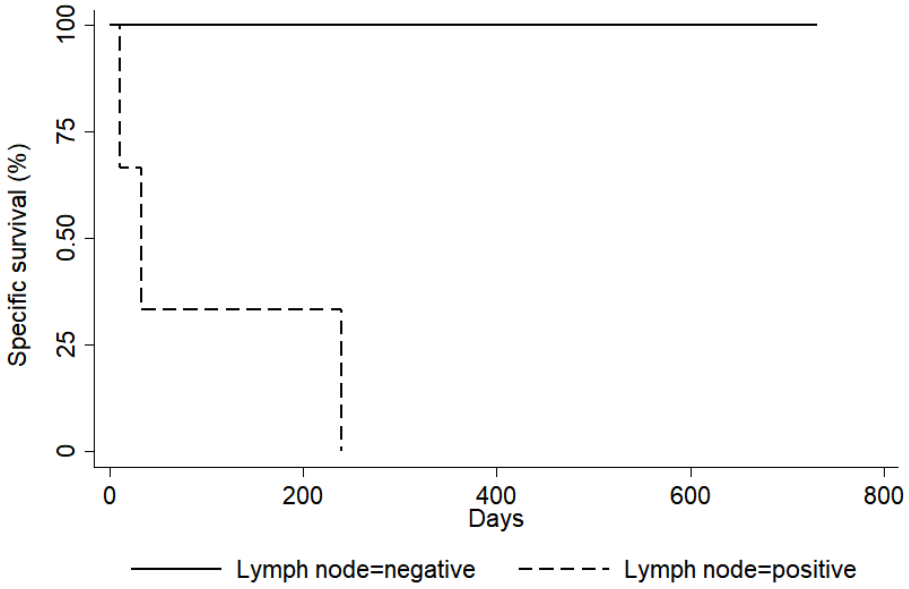

Figure 2. Kaplan Meier specific survival curves of female dogs with carcinosarcomas (n=10). Female dogs with carcinosarcomas stratified by lymph node status. Female dogs with lymph node metastasis demonstrated a lower probability of survival (median of 33 days) than animals lymph node negative (mean of 674 days; log-rank, p=0.010).

DISCUSSION

The frequency of the tumor histological types with mixed proliferations (epithelial/mesenchymal) that we detected in our study sample is similar to other reports in the literature, in which a predominance of carcinomas in mixed tumors and a lower frequency of benign mixed tumors and carcinosarcomas are also observed (Toríbio *et al*., 2012; Nunes *et al*., 2019).

The smaller size of BMT, when compared with CMT and CS, highlights the importance of early surgical treatment of mammary tumors while they are still small. The chances of the tumors being benign and leading to a better prognosis are higher. The results also demonstrated a possible relation between tumor size and the malignant progression of mixed tumors. Some authors have hypothesized that, given enough time, BMT can undergo malignant transformation (Moulton *et al*., 1970; Cassali *et al*., 2017). This hypothesis is based on the analogy of the pleomorphic adenomas that are found in human salivary glands. These are histologically similar to the BMT found in the mammary glands of female dogs, and can evolve into carcinomas ex pleomorphic adenomas if they are not treated early enough (Antony *et al*., 2012).

The high frequency (45.4%) of CMTs larger than 5.0cm stood out in the data and was different from the findings of authors like Nunes *et al*. (2019) who reported that 19.0 % of CMTs were classified as T3. This finding could be due to delays in seeking veterinary care and treatment, which result in more time for the tumors to grow. Tumor size (T > 5cm) has been associated with malignancy, lower expression of progesterone receptors, higher expression of cellular proliferation markers, and shorter survival times (Yamagami *et al*., 1996; Ferreira *et al*., 2009).

Patients with a CS diagnosis showed a lower survival rate and a higher risk of death than those with carcinoma in mixed tumor. This reflects the more aggressive character of neoplasms with sarcomatous differentiation (Benjamin *et al*., 1999; Rasotto *et al*., 2017; Nunes *et al*., 2019). In this group, a higher frequency of other characteristics that are generally associated with tumor aggressiveness, such as larger tumor sizes (Yamagami *et al*., 1996; Ferreira *et al*., 2009) and solid or micropapillary carcinomatous areas (Gamba *et al*., 2017; Nunes *et al*., 2019) was found. Unexpectedly, there was a low frequency of distant metastasis among the female dogs with CS in our study (1/10). This result is also different from those of previous investigations that reported 100% of the female dogs with CS developed pulmonary metastasis by the end of 24 months (Benjamin *et al*., 1999; Rasotto *et al*., 2017).

Lymph node metastasis is an important predictors of clinical outcome in female dogs with malignant mammary tumors (Szczubial and Łopuszynski, 2011; Araújo *et al*., 2016). In this study, metastasis to lymph nodes and clinical stage were associated with shorter survival time and an increased risk for tumor- associated death for female dogs with CTM and CS in univariate analysis. However, these variables failed to maintain their prognostic significance when included in multivariable models. This may be explained by failure to return for image exams during the period of veterinary follow-up, animal death before metastasis or the lack of histopathological confirmation of lymph node status since in 14 cases, the lymph nodes were not surgically removed.

Corroborating previous findings, our study showed that vascular invasion was associated with specific survival time. However, lymphatic invasion lost their prognostic power in the multivariate survival analysis (Rassoto *et al*, 2017, Canadas *et al*., 2019). Other factors as necrosis and larger tumor size, that have been suggested to be indicators of higher tumor, aggressiveness (Hellmen *et al*., 1993; Yamagami *et al*., 1996; Ferreira *et al*., 2009, Nunes *et al*., 2019) were not significantly related to prognosis in this study, which may be explained by the small number of cases studied.

The small number of samples also precluded estimating the risk to tumor- associated death for CTM and CSS groups (Cox proportional hazard multivariate model).

Furthermore, in some cases, follow-up information was obtained through telephone interviews with the animal owners, which may have resulted in incomplete or inaccurate patient data.

As expected, female dogs with CMT had good prognosis with a high survival rate and presented predominantly localized disease. However, some CMT patients in our research were diagnosed with lymph node metastasis, or developed distant metastasis, or died during care. The findings confirm that CMT can exhibit clinically aggressive behavior.

CMTs are histologically heterogeneous and different types of carcinomatous proliferation patterns can be associated with a mesenchymal component. One aspect that we analyzed was the influence of the carcinomatous pattern on the prognosis regarding these neoplasms. Micropapillary carcinomas and solid carcinomas are considered aggressive histological types and have been more closely associated with distant metastasis and death than other types, than other types, including papillary and tubular carcinomas (Gamba *et al*., 2017; Nunes *et al*., 2018). Our findings showed that the aggressive behavior of micropapillary or solid pattern proliferations is preserved as they develop in the mixed tumor since these areas resulted in shorter survival times among female dogs with CTMS.

Besides this, results demonstrated that tumors with micropapillary or solid areas presented higher percentages of necrosis, vascular invasion, lymph node metastasis and clinical stages IV-V than tumors with papillary or tubular proliferation. However, this finding was not statistically significant. The lack of statistical association may have resulted from the small number of cases.

CONCLUSIONS

In conclusion, the results showed that carcinomas in mixed tumors vary in prognosis depending on the carcinomatous proliferation patterns, and this should be taken into consideration in the histological evaluation of this tumor type. Lymph node metastasis proved to be relevant as a prognostic factor, which reinforces the importance of assessing lymph node status.

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