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The prognostic significance of immunophenotypes in canine malignant mammary tumors

[*Significância prognóstica dos imunofenótipos em tumores mamários malignos caninos*]

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ABSTRACT

Canine malignant mammary neoplasms (CMMN) exhibit behavioral variability with the patient survival time depending on several prognostic factors. In the present study, 134 CMMN were selected and different immunophenotypes and their associations with clinical and pathological parameters were identified. The tumors were classified as follows: 46% of luminal B HER2-, 34% of luminal A, 13% of triple-negative, and 7% of luminal B HER2+. Shorter specific survival time were associated with larger tumor sizes (>3.0 cm, HR=1.94; P=0.0209), lymph node metastasis or distant metastasis (HR= 2.82; P <.0001), more aggressive histological types (HR= 7.15, P<0.0001), higher histological grades (HR= 12.97 P=0.011), angiolymphatic invasion (HR=4.68, P<0.0001) and luminal B HER2 - (HR= 3.27, P<0.0001) and luminal B HER2 + (HR= 7.14 P<0.0001) immunophenotypes. In patients with lymph nodal metastasis, shorter survival times were associated with luminal immunophenotype B HER2 + (P=0.003). However, in patients without metastasis, an increased risk of death was associated with the aggressive histological type. In conclusion, the classification in our study allowed us to identify subtypes with different prognoses in canine malignant mammary tumors. Factors such as clinical stage, histological type, luminal B HER2+ subtype, and angiolymphatic invasion were the most important prognostic factors.

Keywords: carcinoma; mammary gland; luminal; triple-negative

RESUMO

Neoplasias mamárias malignas caninas (CMMN) apresentam variável comportamento biológico e o tempo de sobrevida depende de diversos fatores prognósticos. Neste estudo, foram selecionadas 134 CMMN, bem como identificados diferentes imunofenótipos e suas associações com parâmetros clínicos e patológicos. Os tumores foram classificados em: 46% luminal B HER2-, 34% luminal A, 13% triplo negativo e 7% luminal B HER2+. Menores taxas de sobrevida específica foram associadas a tamanhos de tumor maiores (> 3,0cm; HR = 1,94; P = 0,0209), metástases em nodais ou a distância (HR = 2,82; P <0,0001), tipos histológicos mais agressivos (HR = 7,15; P <0,0001), graus histológicos mais elevados (HR = 12,97; P = 0,011), invasão angiolinfática (HR = 4,68; P <0,0001) e aos imunofenótipos luminal B HER2- (HR = 3,27; P <0,0001) e luminal B HER2+ (HR = 7,14; P <0,0001). Em pacientes com estágio avançado, menor sobrevida específica foi associada ao imunofenótipo luminal B HER2+ (P = 0,003). Entretanto, em estágio inicial, um risco aumentado de óbito foi associado a tipos histológicos agressivos. Em conclusão, a classificação utilizada no presente estudo permitiu identificar subtipos com diferentes prognósticos em CMMN. Estágio clínico, tipo histológico, subtipo luminal B HER2+ e invasão angiolinfática foram os fatores prognósticos mais importantes.

Palavras-chave: carcinoma, glândula mamária, luminal, triplo negativo

INTRODUCTION

In breast cancer, the biggest challenge in determining prognosis is the heterogeneity of this tumor type. Tumors of similar histological types, clinical stages, and degrees of differentiation may have different prognoses and therapeutic responses (Yersal *et al.,* 2014). For this reason, different technologies have been used to stratify breast cancer types, according to molecular similarities. Use of technology such as DNA microarrays to evaluate gene expression has made it possible to classify human breast cancer into the following molecular subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2-positive), and basal-like (Perou *et al*., 2000).

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Due to the difficulty in performing techniques such as DNA microarrays in the laboratory, immunohistochemical panels have been proposed to identify immunophenotypes in human breast cancer (Nielsen *et al*., 2004, Blows *et al*., 2010, Goldshirsch *et al*., 2011,2013). Since 2011, the immunohistochemical analyses of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and cell proliferation indexing (Ki67) have been adopted to classify early breast cancer into the following clinical and pathologic subtypes genetically determined (Goldshirsch *et al*., 2011, 2013): luminal A, luminal B (HER2‑positive), luminal B (HER2‑negative) and triple‑negative. In addition, panel complementation with analysis of cytokeratin [CK]5/6 and epidermal growth factor receptor (EGFR) expression has been shown to accurately identify basal-like tumors, a subtype that expresses basal and myoepithelial markers (Nielsen *et al*., 2004; Blows *et al*., 2010).

Similar to human breast tumors, canine malignant mammary neoplasms (CMMNs) belong to a heterogeneous group in terms of morphology and biological behavior (Gama *et al*., 2008; Rasotto *et al*., 2017). Immunohistochemical markers have been used for molecular classification of canine mammary tumors (Gama *et al*., 2008; Sassi *et al*., 2010; Kim *et al*., 2013; Im *et al*., 2014; Abadie *et al*., 2018; Varallo *et al*., 2019) due to the great interest of researchers in broadening the understanding of tumors' biology and the prognoses of CMMNs. However, there are differing reports regarding the distribution and prognostic effect of these immunophenotypes. This may be related to differences among the immunohistochemical panels used to classify neoplasms or distinct cut-off scores for determining the positivity of biomarkers (Gama *et al*., 2008; Sassi *et al*., 2010; Abadie *et al*., 2018). Although molecular classification based on ER, PR, HER2, and Ki67 immunohistochemical measures has been already adopted for molecular categorization of women's breast cancer (Goldshirsch *et al*., 2011, 2013), a few studies have focused on the assessment of such markers in canine mammary tumors. The present research's approach is important to validate a canine comparative model and may also provide prognostic information on female dogs with mammary tumors. In this sense, we aimed to evaluate: 1) the distribution of CMMN immunophenotypes and organize them in an adapted schema based on the immunohistochemical classification of human breast cancer; 2) the association between CMMN immunophenotypes and clinical and pathological features; 3) the prognostic value of clinical and pathological features and CMMN immunophenotypes in initial and advanced stages of the disease.

PATIENTS AND METHODS

The study included patients on a follow-up visit after their first appointment at the Oncology Sector of the Veterinary Hospital of the Federal University of Minas Gerais (UFMG), Brazil, between 2011 and 2015. The analysis of surgically resected mammary tumor samples and corresponding lymph nodes was performed by the Laboratory of Comparative Pathology at the Institute of Biological Sciences of UFMG.

The selection criteria were: 1) diagnosis of carcinoma in mixed tumors, invasive papillary carcinoma, tubular carcinoma, malignant adenomyoepithelioma, solid carcinoma, micropapillary carcinoma, pleomorphic lobular carcinoma or carcinosarcoma; 2) available formalin-fixed paraffin-embedded blocks for immunohistochemistry experiments 3) available follow-up data 4) Patients with a minimum follow-up time from 2 years (24 months) 5) no history of other malignancies and 6) no adjuvant chemotherapy.

Clinical features were obtained from medical records and these included patient age (≤ 10 and >10 years), breed (crossbred or purebred), reproductive status (intact or spayed), history of skin ulceration or adherence and tumor size (≤ 3.0 and >3.0 cm). Pathological features were assessed by reviewing hematoxylin and eosin-stained tissue sections and included examination of regional metastasis, lymph node involvement, angiolymphatic invasion, histological type, and histological grade.

The hematoxylin and eosin-stained slides were reviewed in each case to confirm the original diagnosis, following the histological classification criteria proposed by the World Health Organization (Misdorp *et al*., 1999) and Cassali *et al*. (2014, 2017).

For the present study, one tumor was selected per animal. When multiple malignant mammary tumors were present in a single dog, the tumor with the most aggressive histology (i.e. solid carcinoma, carcinosarcoma, micropapillary carcinoma, pleomorphic lobular carcinoma, which present the highest disease aggression and histological grade) was selected for analysis. All tumor slides were reviewed, and the most representative sections were chosen for each specimen.

Tumors were graded according to the Nottingham system (Elston and Ellis, 1991). Tumor metastasis was diagnosed histologically in lymph nodes providing drainage to the affected mammary gland (inguinal or axillary). The presence of neoplastic cells in lymphoid parenchyma or distributed along lymphatic sinuses were considered features of metastases. The presence or absence of tumor necrosis and angiolymphatic invasion were also considered.

All cases were classified according to the Tumor-Node-Metastasis (TNM) system established by the World Health Organization (WHO) for canine mammary gland tumors (Owen, 1980) and adapted by Sorenmo *et al*. (2013). Tumor staging was based on tumor diameter and pathological parameters (T_1: 0-3cm; T_2: 3-5 cm; T_3: >5 cm), while neoplastic involvement of regional lymph nodes (N_0: non-metastatic; N_1: metastatic) was defined histopathologically. Distant metastasis (M_0: non-metastatic; M_1: metastatic) was determined through thoracic x-rays and abdominal ultrasound examination before surgery. Patients were clinically assessed during the first visit and imaging exams were repeated for a minimum period of at least every 3 months during the 2-year follow-up.

Consecutive sections of tumors (4µm thick) were prepared and immunohistochemical reactions were performed using the streptavidin-biotin-peroxidase complex method and a commercial anti-mouse/anti-rabbit detection system (Novolink Polymer Detection System, Leica Biosystems, Newcastle upon Tyne, UK), according to the manufacturer's instructions. Antigen retrieval for estrogen receptor (ER), progesterone receptor (PR), Ki67, and HER2 was performed using steam heat (Pascal®) with citrate pH 6.0 (Dako Cytomation Target Retrieval Solution, Dako, Glostrup, Denmark). For the cytokeratin 5/6 (CK5/6) antibodies, retrieval was done using steam heat (Pascal®) with Trilogy® retrieval buffer (Cell Marque, Rocklin, CA, USA). For the epidermal growth factor receptor (EGFR), enzymatic antigen retrieval with pepsin occurred in an oven drier at 37°C.

All sections were incubated with the appropriate primary antibody for 16 h in a humidified chamber at 4°C: ER (1:50, clone 1D5, Dako), PR (1:50, hPRa2, Neomarkers, Fremont, CA, USA), HER2 (1:200, polyclonal, Dako), Ki67 (1:50, MIB-1, Dako), EGFR (1:50, clone 31G7, Invitrogen, Carlsbad, CA, USA) and CK5/6 (1:50, clone D5/16/B4, Dako). Immunoreactivity was visualized with chromogen 3'-diaminobenzidine (DAB Substrate System, Dako, Carpinteria, CA, USA) and counterstained with Mayer's Hematoxylin. Breast tumor sections from women with HER2- positive breast cancer were used as positive control in each reaction. Negative controls were assessed with normal serum as the primary antibody. All antibodies had been previously documented as suitable for the detection of epitopes in canine tissues (Araújo *et al*., 2016).

To determine the cell proliferation rate (Ki67) and ER and PR positivity, the number of positive nuclei in a total of 500 neoplastic cells in hotspot areas was counted through manual image analysis, with ImageJ software (National Institute of Health, Bethesda, MD, USA). In this analysis, >10% was considered positive for ER and PR. A value of Ki67 ≥20% was used to classify cases with high cell proliferation rate.

A scoring system established by the American Society of Clinical Oncology, College of American Pathologists (ASCO/CAP) (Wolff *et al*., 2013) was used to determine HER2 expression: (0 = no membrane staining or incomplete and faint/barely perceptible membrane staining in ≤10% of tumor cells; 1 + = incomplete and faint/barely perceptible membrane staining in ≥10% of tumor cells; 2 + = incomplete and/or weak/moderate membrane staining in >10% of tumor cells or complete and intense membrane staining in ≤10% of tumor cells; and 3 + = complete and intense membrane staining in >10% of tumor cells). In our study, specimens with scores of 0, 1+, and 2+ were regarded as negative, whereas a score of +3 was defined as positive.

A qualitative evaluation of CK5/6 cytokeratin expression was performed, and the cytoplasmic staining of neoplastic cells was considered positive. EGFR expression was assessed using criteria adapted from the HER2 assessment, which states that specimens with scores of 0, 1+, and 2+ are considered negative and those with a score of 3+ are considered positive (Wolff *et al*., 2013).

A classification like the one proposed by St. Gallen Consensus (Goldshirsch *et al*., 2011, 2013), which stratifies tumors as luminal, HER2-overexpressing, or triple-negative, was used. Luminal tumors were subdivided into luminal A, luminal B-HER2-negative, and luminal B-HER2-positive, and the cutoff for the cell proliferation rate (Ki67 of 20%) and HER2-overexpression were considered.

The subtypes are classified as follows: luminal A: ER and/or PR-positive, HER2-negative and low Ki67 (<20%); luminal B HER2-negative: ER and/or PR-positive, HER2-negative and high Ki67 (≥20%); luminal B HER2-positive: ER and/or PR-positive and HER2-positive and any Ki67; HER2-overexpressing (non-luminal): ER and PR-negative and HER2-positive; and triple-negative: ER, PR, and HER2 negative. The ideal cutoff value for Ki67 was defined statistically through disease-specific survival. The 10, 15, and 20% Ki67 cut-off points were tested against disease-specific survival (SS) and only the cutoff value of 20% was significantly associated with a shorter SS. The triple-negative cases were thus defined as the absence of positivity for ER, PR, and HER2 and classified as basal cases when positive for CK5/6 and/or EGFR.

The follow-up time ranged from 2 years (24 months) to 5.8 years. Specific survival (SS) was estimated from the date of mastectomy to the date of death from disease progression. All surviving patients at the end of follow-up were censored at the last date in their medical records. Patients lost to the follow-up exercise were analyzed up to the last date in their records. Patients who died from causes unrelated to mammary cancer were censored at the date of death. The starting time of observation for each individual (T_0) was defined as the date of surgery for the removal of the mammary tumor.

To better establish the prognostic value of the clinical and pathological variables of interest (i.e., age, tumor size, clinical stage, histological type, histological grade, angiolymphatic invasion, ulceration, necrosis, adherence, and immunophenotypes), univariate and multivariate analyses were performed to analyze SS. For the analysis, animals were stratified into two groups according to the clinical stage of their disease: $T_{1,2,3}N_0M_0$ and $T_{1,2,3}N_1M_0$.

Survival function was estimated through Kaplan–Meier analysis and differences in survival were compared with the log-rank test. Values were considered statistically significant when $P < 0.05$. A Cox proportional-hazards regression analysis was performed to identify potential hazard ratios (HRs) associated with SS and evaluate the prognostic value of the study variables.

Multivariate analysis included only those variables with P-value of 0.05, or lower in the univariate analysis (log-rank testing). After this step, all $p<0.05$ variables were included in the analysis and selected through a process of "backward elimination." The significance of the parameters of the reduced models and the final model was verified using a likelihood ratio test and the proportionality of the Cox models was verified using Schoenfeld residuals. The final model included all $p<0.05$ variables. Two Cox proportional-hazard multivariate models were analyzed and included all clinical and pathological variables. The first model included only cases of patients without metastasis ($T_{1,2,3}N_0M_0$) while the second model was performed with data of patients with lymph node involvement ($T_{1,2,3}N_1M_0$). All analyses were performed using Stata software, version 14.0 (Stata Corp, College Station, TX, USA).

Ethical approval for all procedures was obtained from the Animal Experimentation Ethics Committee of the Federal University of Minas Gerais (UFMG) - (approval number 366/2016). Similarly, the present study was performed according to their guidelines.

RESULTS

The cohort was composed of 134 female dogs with malignant mammary tumors. The mean age at diagnosis was 11.38±2.75 years old (range: 4 to 17 years of age). The malignant mammary tumors included 47 (35%) cases of less aggressive neoplasms: malignant adenomyoepithelioma (9 cases, 19%), invasive

papillary carcinoma (6 cases, 13%), tubular carcinoma (9 cases, 19%), and carcinoma in mixed tumors (23 cases, 49%). There were 87 (64%) cases of more aggressive neoplasms: micropapillary carcinoma (31 cases, 36%), solid carcinoma (30 cases, 34%), carcinosarcoma (21 cases, 24%), pleomorphic carcinoma (5 cases, 6%). According to the histological grade, CMMN were classified into 13% grade I (12 cases), 45% grade II (42 cases) and 42% grade III (39 cases). Presence of angiolymphatic invasion was observed in 67% of cases. Four immunophenotypes were identified: luminal A, 34% (45/134), luminal B HER2-, 46% (62/134), luminal B HER2+, 7% (9/134) and triple-negative, 13% (18/134). No significant association was found between the clinical and pathological features of the patients with luminal and non-luminal CMMN. The summary of this data is available in the Table 1.

Table 1. The clinical and pathological characteristics of female dogs with malignant mammary neoplasms, according to luminal or non-luminal immunophenotypes.

Variables	n	Luminal	Non-Luminal	P-value
Age (mean ± SD)				
Unknown				
Breed				
Crossbred	37 (29)	30 (27)	9 (56)	0.169
Purebred	90 (71)	81 (73)	7 (44)	
Unknown	7			
Reproductive status				
Intact	57 (58)	47 (54)	10 (83)	0.066
Spayed	42 (42)	(46)	2 (17)	
Unknown	35			
Histological type				
Carcinoma in a mixed tumor	23 (17)	20 (17)	3 (17)	0.555
Tubular carcinoma	9 (7)	8 (7)	1 (6)	
Solid carcinoma	30 (22)	27 (23)	3 (17)	
Micropapillary carcinoma	31(23)	28 (24)	3 (17)	
Pleomorphic lobular carcinoma	5 (4)	3 (3)	2 (11)	
Malignant adenomyoepithelioma	9 (7)	7 (6)	2 (11)	
Carcinosarcoma	21 (16)	17 (15)	4 (22)	
Invasive papillary carcinoma	6 (4)	6 (5)	0	
Histological grade				
I	12 (13)	11 (13)	1 (9)	0.329
II	42 (45)	39 (47)	3 (27)	
III	39 (42)	32 (39)	7 (64)	
Unknown	41			
Stage (TNM)				
I ($T_1N_0M_0$)	21 (16)	20 (17)	1 (6)	0.624
II ($T_2N_0M_0$)	12 (9)	11 (9)	1 (6)	
III ($T_3N_0M_0$)	31 (23)	25 (22)	6 (35)	
IV ($T_{1,2,3}N_1M_0$)	61 (46)	53 (46)	8 (47)	
V ($T_{1,2,3}N_{0,1}M_1$)	7 (5)	6 (5)	1 (6)	
Unknown	2			
Tumor size				
T_1 (<3.0 cm)	40 (30)	38 (33)	2 (12)	0.189
T_2 (3.0 – 5.0 cm)	21 (16)	18 (16)	3 (18)	
T_3 (>5.0 cm)	71 (54)	59 (51)	12 (70)	
Unknown	2			
Angiolymphatic invasion				
Present	83 (67)	70 (65)	13 (76)	0.272
Absent	41 (33)	37 (34)	4 (25)	
Unknown	10			
Skin ulceration				
Absent	113 (85)	100 (87)	13 (72)	0.104
Present	20 (15)	15 (13)	5 (28)	
Unknown	1			

Results of Kaplan-Meier survival analyses, log-rank tests, and hazard ratio (HR) in relation to specific survival are listed in Table 2, with variables. Shorter specific survival was associated with larger tumor sizes (>3.0 cm, HR=1.94; P=0.024), lymph node metastasis or distant metastasis ($T_{1,2,3}N_1M_{0,1}$) HR= 2.82; P <.0001), more aggressive histological types (HR= 7.15, P<0.0001), higher histological grades (II-III versus I, HR= 12.97 P=0.011), angiolymphatic invasion (HR=4.68, P<0.0001) and luminal B HER2 - (HR= 3.27, P<0.0001) and luminal B HER2 + (HR= 7.14 P<0.0001) immunophenotypes.

Table 2. Univariate analyses of specific survival (Kaplan-Meier) in relation to clinical and pathological variables in female dogs with CMMN

Variables	Specific survival			
	Median SS	P-value	HR	CI
Age				
<10 years	329	Reference*		
>10 years	320	0.640	1.12	0.68-1.83
Tumor size				
< 3 cm	950	Reference*		
≥ 3 cm	283	0.024	1.94	1.09-3.45
Clinical stage				
I-III	-	Reference*		
IV-V	163	<0.001	2.82	1.68-4.75
Histological type				
Less aggressive	-	Reference*		
More aggressive	187	<0.0001	7.15	3.34-15.29
Histological grade				
I	-	Reference*		
II- III versus I	267	0.011	12.97	1.77-94.61
Angiolympathic invasion				
Absent	625	Reference*		
Present	140	<0.0001	4.68	2.78-7.88
Ulceration				
Absent	368	Reference*		
Present	180	0.613	1.18	0.61-2.25
Adherence				
Absent	404	Reference*		
Present	294	0.286	1.34	0.78-2.30
Immunophenotypes				
Luminal A	950	Reference*		
Luminal B HER2-	207	<0.0001	3.27	1.73-6.17
Luminal B HER2+	120	<0.0001	7.14	2.85-17.87
Triple negative	461	0.127	1.94	0.82-4.56

*The Reference subgroup is given a hazard ratio of 1.00. In comparison, other subgroups are associated with shorter SS if their hazard ratio is higher than 1.00. A subgroup with HR >1.00 and P<0.05 is associated with shorter SS than the Reference subgroup.

Prognostic factors associated with CMMN in patients without metastasis - $T_{1,2,3}N_0M_0$.

Univariate analyses of specific survival showed that tumor size >3.0 cm (HR= 6.64, CI: 1.51-29.18, P=0.012), clinical-stage II (HR= 7.99, CI: 1.60-40.00, P= 0.011), clinical-stage III (HR= 6.05, CI: 1.32-27.80, P= 0.020), more aggressive mammary tumors (HR= 5.61, CI: 1.61-19.53, P=0.007) and the luminal B HER2- immunophenotype (HR= 4.08, CI: 1.28-12.96, P=0.017) were associated with higher risk of death (Table 3). In the multivariate analysis, an increased risk of death was associated with T_2 and T_3 tumor sizes and aggressive histological types: micropapillary carcinoma, solid carcinoma, pleomorphic carcinoma, grade III tubular carcinomas, and carcinosarcomas (Table 3).

Prognostic factors associated with CMMN in patients with lymph node metastasis – $T_{1,2,3}N_1M_0$. The multivariate analysis regarding specific survival and $T_{1,2,3}N_1M_0$ disease showed association between angiolymphatic invasion (HR=3.47. CI: 1.53-7.88, P=0.003), the luminal B HER2+ immunophenotype (HR=3.51, IC: 1.11-11.01, P=0.031) and higher risk of death (Table 4).

Associations between immunophenotypes and specific survival – To determine whether immunophenotypes influence survival, we investigated specific survival in a cohort of 66 dogs without metastasis ($T_{1,2,3}N_0M_0$) that were treated only with surgery. Although a longer specific survival time was associated with the luminal subtype A (Figure 1A), no significant difference was found in the survival times among immunophenotypes (P=0.06). However, when assessing specific survival and immunophenotypes in patients with lymph nodal metastatic, we observed a shorter survival time associated with luminal B HER2 + immunophenotype (P=0.003) (Figure 1B).

Table 3. Univariate and multivariate Cox proportional-hazards regression analyses comparing the clinical and histopathological aspects of CMMN in female dogs without metastasis - **$T_{1,2,3}N_0M_0$**

Variables	HR	(95% CI)	P-value
	Univariate analysis - SS		
Age			
<10 years versus >10 years	1.50	0.57-3.92	0.402
Tumor size			
< 3 cm versus ≥ 3 cm	6.64	1.51-29.18	0.012
Clinical stage			
II versus I	7.99	1.60-40.00	0.011
III versus I	6.05	1.32-27.80	0.020
Histological type			
More aggressive tumors versus less aggressive tumors*	5.61	1.61-19.53	0.007
Histological grade			
II versus I	1.39	0.12-15.56	0.788
III versus I	7.55	0.93-60.99	0.058
Angiolympathic invasion			
Absent versus present	4.28	0.91-20.15	0.065
Ulceration			
Absent versus present	1.65	0.47-5.76	0.428
Adherence			
Absent versus present	2.08	0.77-5.60	0.145
Immunophenotypes			
Luminal B HER2- versus Luminal A	4.08	1.28-12.96	0.017
Luminal B HER2+ versus Luminal A	5.17	0.54-48.90	0.151
Triple negative versus Luminal A	3.52	0.87-14.13	0.076
Variables	HR	(95% IC)	P-value
Histological type*	*Multivariate analysis - Specific survival*		
More aggressive tumors versus less aggressive tumors	5.13	1.43-18.37	0.012
Tumor size**			
T_2 versus T_1	7.58	1.48-38.67	0.015
T_3 versus T_1	5.72	1.23-26.59	0.026

*Less aggressive types: carcinomas in mixed tumors, invasive papillary carcinoma, grade I or II tubular carcinomas, and malignant adenomyoepithelioma. More aggressive types: solid carcinoma, grade III tubular carcinoma, micropapillary carcinoma, pleomorphic lobular carcinoma, and carcinosarcoma. **T_1:<3.0cm, T_2: 3.0-5.0cm and T_3>5.0cm.

Table 4. Univariate and multivariate Cox proportional-hazards regression analyses comparing the clinical and histopathological aspects of CMMN in female dogs with lymph node metastasis - $T_{1,2,3}N_1M_0$

Variables	HR	(95% CI)	P-value
	Univariate analysis - SS		
Age			
<10 years versus >10 years	1.20	0.63-2.28	0.560
Tumor size			
< 3 cm versus ≥ 3 cm	1.24	0.63-2.41	0.522
Histological type			
More aggressive tumors versus less aggressive tumors*	9.18	3.19-26.42	<0.001
Histological grade			
II versus I			
III versus I			
Angiolympathic invasion			
Absent versus present	3.86	1.86-7.99	<0.001
Ulceration			
Absent versus present	1.15	0.53-2.50	0.715
Adherence			
Absent versus present	1.33	0.68-2.61	0.391
Immunophenotypes			
Luminal B HER2- versus Luminal A	2.09	0.96-4.57	0.063
Luminal B HER2+ versus Luminal A	6.13	2.10-17.86	0.001
Triple negative versus Luminal A	1.32	0.44-3.97	0.616
Variables	HR	(95% CI)	P-value
Angiolympathic invasion	*Multivariate analysis - SS*		
Absent versus present	3.47	1.53-7.88	0.003
Immunophenotypes			
Luminal B HER2- versus Luminal A	1.06	0.44-2.58	0.885
Luminal B HER2+ versus Luminal A	3.51	1.11-11.01	0.031
Triple negative versus Luminal A	0.99	0.32-3.05	0.993

*Less aggressive types: carcinomas in mixed tumors, invasive papillary carcinoma, grade I or II tubular carcinomas, and malignant adenomyoepithelioma. More aggressive types: solid carcinoma, grade III tubular carcinoma, micropapillary carcinoma, pleomorphic lobular carcinoma, and carcinosarcoma.

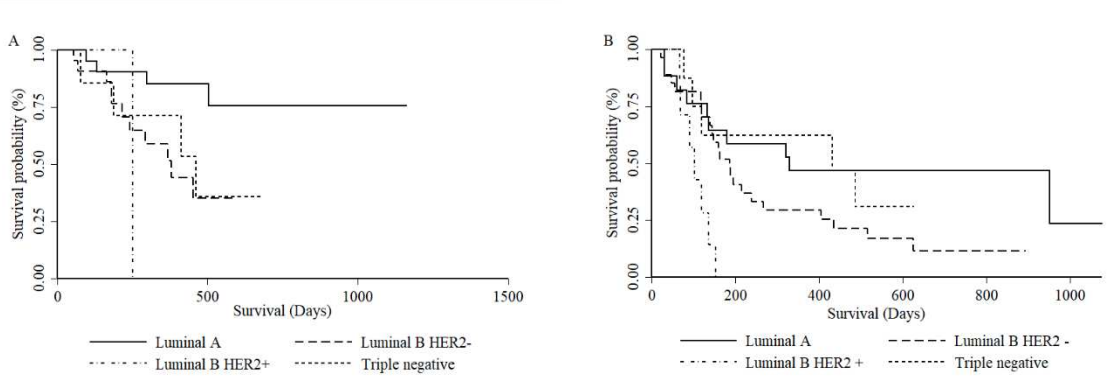

Figure 1: Kaplan-Meier curve of female dogs with mammary tumors according to the immunophenotype, all treated with surgery alone. (A) Patients without metastasis ($T_{1,2,3}N_0M_0$), n= 66 cases, P=0.06, (B) Patients with lymph node metastasis ($T_{1,2,3}N_1M_0$), n=61 cases, P=0.003.

DISCUSSION

The present study identified the distribution and prognostic value of immunophenotypes in CMMNs using immunohistochemical panels and the St Gallen classification recommendations (Goldshirsch *et al*., 2011). This classification was important for the clarification of the heterogeneous nature of mammary neoplasms in patients with the same clinical stage but different clinical disease progression. Ours is the first study to subdivide the luminal subtypes into three categories: luminal A, luminal B HER2- and luminal B HER2 +. According to such classification, the most frequent immunohistochemical subtype was luminal B HER2 – followed by luminal A. Gama *et al*. (2008) used a panel of five markers (ER, HER2, CK5, p63, and P-cadherin) to detect the following phenotypes: luminal A (44.8%), luminal B (13.5%), HER2-overexpressing (8.3%), basal-like (29.22%) and null/negative phenotype (4.2%). Upon using ER, PR, HER2, CK5/6 and CK14 markers, Sassi *et al*. (2010) only identified luminal A (19%), luminal B (49%), and basal-like (22%) subtypes. Subsequently, six antibodies were used to classify luminal A (44%), luminal B (22.6%), HER2-overexpressing (5.7%), basal-like (24.5%), and normal breast-like subtypes (3.1%): ER, HER2, CK14, P63 and vimentin (Im *et al.,* 2014). All these authors observed higher frequency of luminal tumors, corroborating the results obtained in our study. However, varying criteria for the classification, selection, and quantification of immunohistochemical markers make it difficult to compare the results of different immunophenotype studies on canine mammary neoplasms.

In previous research, hormone receptor positivity (ER and/or PR) and HER2 overexpression were the criteria used to classify the luminal B subtype (Gama *et al*., 2008, Sassi *et al*., 2010), which corresponds to the luminal B HER2 + subtype described in our study, albeit with distinct terminology. Nevertheless, our results observed showed a lower frequency of this subtype compared to previous studies. We attributed this finding to the classification criteria we used, based on those established for breast cancer in women, and according to which only 3+ cases were considered positive for HER2. The Ki67 score, with a cutoff of 33%, was recently considered for molecular classification of CMMNs in female dogs (Abadie *et al*., 2018). Tumors were classified as luminal A (ER and/or PR+, HER2, Ki67 <33%) and luminal B (ER and/or PR+, HER2, Ki67 ≥33%). Our findings suggest that a Ki67 cutoff point of 20% can be used to discriminate between and predict the prognosis of the luminal A and luminal B HER2- subtypes, since female dogs with Ki67 score greater than 20% presented lower survival rates.

The absence of HER2-overexpressing phenotype in our study population can be explained by the high frequency of positivity for ER and/or PR and by our methodology, according to which only 3+ cases were considered HER2-positive. These results corroborate the findings of Abadie *et al*. (2018), who also did not detect HER2-overexpressing cases in their study population while using a methodology similar to ours. While other authors reported the HER2-overexpressing subtype in 8.3% and 5.7% of evaluated canine mammary tumors, they considered tumors that expressed HER2 with incomplete and/or weak/moderate membrane staining in >10% cells or with strong and complete membrane staining in ≤10% of the tumor cells (2+) as positive (Gama *et al.,* 2008, Im *et al.,* 2014). According to Peña *et al*. (2014), there should be a standardization for canine mammary tumors that only considers cases with a 3+ score (complete expression in more than 10% of neoplastic cells).

In the univariate analysis, larger tumor sizes, lymph node metastasis or distant metastases, more aggressive histological types, higher histological grades, angiolymphatic invasion, and the luminal B HER2 - and luminal B HER2 + immunophenotypes were associated with shorter specific survival. These results are of extreme importance for the definition of prognosis and adjuvant therapy for veterinary doctors and oncologists. Araújo *et al.* (2016) observed that angiolymphatic invasion, high Ki67 rates, and larger tumor sizes were associated with shorter overall survival of female dogs with mammary carcinomas.

The main strength of our study is that it evaluated the prognostic factors of female dogs without metastasis and with lymph node metastasis separately, since these data may allow professionals to better predict disease progression and assist oncologists in developing

a treatment plan. In patients without metastasis, histological type and tumor size were factors associated with shorter SS times. Previous studies have shown worse outcomes associated with aggressive histological types such as carcinosarcoma, micropapillary carcinoma, and solid carcinoma, as well as >3.0cm tumors (Ferreira *et al.,* 2009; Nunes *et al.,* 2018). When determining prognostic factors for patients in advanced stages, testing positive for angiolymphatic invasion and a luminal B HER2+ immunophenotype can inform predictions regarding disease progression. These data are important to confirm mammary tumor heterogeneity and highlight immunophenotyping as an auxiliary tool for patients with metastatic disease.

Immunophenotypic classification is an important predictive and prognostic factor in breast cancer in women but individualized targeted therapies for each immunophenotype have still not been used in veterinary oncology. ER and PR expression are associated with cell differentiation and, in comparison with normal mammary glands, decrease progressively from hyperplasic/dysplastic lesions, benign neoplasms and malignant neoplasms (Chang *et al*., 2005). In canine mammary tumors, high ER and PR expression suggest that using antiestrogen therapy (Tamoxifen) is a possibility. However, this drug has been associated with side effects such as vulvar edema, incontinence, urinary tract infection, endometritis, pyometra, and ovarian cysts (Tavares *et al*., 2010). Given the absence of antiestrogens for female dogs, we suggest that ovariohysterectomies (OHE) may be beneficial in inhibiting hormonal stimulation in female dogs with hormone-receptor-positive neoplasms. This recommendation can be justified by the fact that, in neoplastic cells, the interaction between estrogen and the receptor stimulates the release of growth factors, which leads to increased cell proliferation (Sonremo *et al.,* 2013). Increases in Ki67 expression and decreases in ER and PR expression are associated with a worse prognosis and suggest the need for adjuvant chemotherapy (Cassali *et al.,* 2014). Although HER2 expression was a negative prognostic factor, therapy with anti-c-erbB2 monoclonal antibodies has still not been used in veterinary oncology.

CONCLUSIONS

Using the St Gallen classification allowed us to identify subtypes with different prognoses in CMMNs. In patients without metastasis, histological type and tumor size were associated with shorter SS times. In patients with lymph node metastasis, the luminal B HER2+ subtype and angiolymphatic invasion were the most important prognostic factors.

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