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Canine mammary carcinomas: influence of histological grade, vascular invasion, proliferation, microvessel density and VEGFR2 expression on lymph node status and survival time

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Abstract

Spontaneous invasive non-inflammatory canine mammary carcinomas (CMC) and their regional lymph nodes (LN) were analysed ($n = 136$). Histological grade (HG) and vascular invasion (VI) in the tumours and lymph node status were recorded. Proliferation index (PI), microvessel density (MVD) and vascular endothelial growth factor receptor 2 (VEGFR2) expression were estimated using anti-proliferating cell nuclear antigen (PCNA), anti-von Willebrand factor and anti-Flk-1, respectively. Eighteen months follow-up was performed (34 bitches). Tumours of different grades showed differences regarding PI, Flk-1/integrated optical density (Flk-1/IOD) and MVD. Every feature showed significant association with LN status through bivariate analyses. From multivariate analyses, VI and Flk-1/IOD were selected to predict LN status. Data revealed that the probability of a CMC-bearing bitch to remain alive at 1, 4, 5 and 14–18 months was 0.91, 0.87, 0.81 and 0.77, respectively. Besides LN status, VI was the only feature positively correlated with survival time, although a trend to shorter survival of animal patients bearing high expressing VEGFR2 CMC was noted.

Keywords
angiogenesis, dog, mammary gland, neoplasia, prognosis

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Introduction

Mammary tumours, most of which are malignant, are the most common neoplastic disease in intact female dogs. This is a disease of middle-aged or older bitches (8–11 years).[1–4] The morbidity rate varies among countries with different practices about early neutering, and is higher where bitches are not spayed at an early age.[1–3] Canine mammary carcinomas (CMC), malignant tumours arising from epithelial components of the mammary gland, constitute the majority of the mammary gland tumours.[2,4–6] Carcinoma cells show malignant traits, such as invasion of surrounding tissues, entry into and survival in

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the bloodstream, extravasation and survival at the target organ, which in turn lead to overt clinical metastases, deterioration and death of the animal. The above-mentioned malignant traits depend on the modification of otherwise normal cellular processes, mainly cell proliferation, adhesion, migration and matrix remodelling, all of which are modulated by signalling events.[4,7] Lung metastases are the main cause of death of CMC-bearing bitches.[4]

CMC have heterogeneous clinical evolution in terms of rate of metastases, recurrence, quality of life and time to lethal outcome. Increased knowledge is needed to better predict neoplastic behaviour and develop therapeutic practices. Large efforts are being made in the veterinary research community, in an attempt to better understand the cellular and molecular aspects of CMC progression in dogs, and the features in common with humans and rats, which have been exhaustively studied. While the public shows increasing commitment to pet attention and expectation of veterinary care, new therapeutic protocols are being designed and successfully tested in veterinary oncology to complement standard surgical procedures. Among other developments, the progress made in human oncology concerning molecular targeted drugs encourages veterinary oncologists in their practice.[8,9] Several researchers have updated the knowledge on prognostic aspects on CMC and feline mammary carcinomas (FMC) by means of contributions highly heterogeneous in type and methodological conditions.[2,6,10–36]

Lymph node (LN) status is the most important predictor of clinical outcome both in human and animal patients with mammary tumours.[1,3,5,17,27,34,37–42] However, knowledge on the prognostic significance of histological grade (HG), vascular invasion (VI), proliferation index (PI) and angiogenesis in LN metastasis development and survival of canine patients remains heterogeneous.

HG is a score assigned to a tumour according to a semiquantitative assessment of the degree of differentiation, based on morphological analysis. Several articles have been published regarding the prognostic relevance of HG in mammary tumours of dogs[14,18,19,22,23,25,28–30,32–34,40,41,43–50] and cats.[21,51–53] However, the notion of 'HG' does not always refer to the same or equivalent features or processes. For instance, in most of the references cited here, HG assesses tubular formation, nuclear pleomorphism and mitotic rate, although with subtle differences, whereas in other works[19,40,43,44,54] HG also assesses VI by tumoural emboli or even LN status. Among grading systems, Elston and Ellis numerical method for breast cancer (known as the Nottingham method, and based on the Scarf–Bloom–Richardson method),[55] as well as Misdorp's and Peña's slightly different canine adapted methods,[4,14] are the most prevalent. Higher grade has been associated with shorter survival in some articles.[34,41,48]

Among the methods available to quantify the proliferation rate, specific antigens are usually identified in paraffin-embedded tissues. One of the most used antigens is the proliferating cell nuclear antigen (PCNA), a sliding clamp protein essential for DNA replication.[56] PCNA activation through phosphorylation correlates with poor survival of women, and PCNA index seems to be predictive in women's breast cancer.[57] However, the extent of the impact of proliferation on tumour spread, metastasis development or deaths related to CMC or FMC remains controversial.[10,12,21,31,40,44,51,58–60]

As very few carcinoma cells can survive beyond their microenvironment so as to cause secondary tumours, the mere presence of tumour cells in the vessel lumen does not guarantee that they will give rise to a metastatic population. Hence, although VI by neoplastic emboli is often observed, it is not known whether their presence is useful to predict metastasis development or overall survival in small species.[12,19,21,28,34,48]

Angiogenesis is the most important mechanism of vascularisation in mammary carcinomas, although some malignant tumours are known to acquire their blood supply by means of other mechanisms, such as vascular mimicry or vascular cooption.[30,61] The angiogenic process may be quantified either by estimating the expression of outstanding molecules or by measuring microvessel density (MVD). Among the myriad of mediators involved in signalling pathways leading to angiogenesis, the vascular endothelial growth factor (VEGF) family, as a key regulator of normal and

pathological angiogenesis, is the focus of basic and pharmacological research.[62] Particularly, VEGF receptor 2 (VEGFR2), also known as foetal liver kinase-1 (Flk-1), mediates most of the downstream effects of the growth factors concerning promotion of mitosis, enhancement of survival and migration of endothelial and non-endothelial cells (neurons, astrocytes and smooth myocytes). VEGFR2 is a receptor-type tyrosine kinase (RTK) with a catalytic domain within the cytoplasmic region, a hydrophobic transmembrane domain and seven extracellular binding domains.[62,63] The report of *in vitro* and *in vivo* VEGFR2 expression in carcinoma cells generated great interest in the autocrine function of this system.[22,50,64,65]

The method most commonly used to assess intratumoural MVD is a subjective enumeration of stained microvessels within areas of denser microvasculature at a defined magnification. It has been reported that MVD is higher in LN (+) breast tumours,[38] although the clinical significance of this finding has not yet been established. In female dogs, most studies have been performed in very few carcinomas with inconclusive results.[18,24,32,66–68]

The purpose of the present study was to determine whether HG, VI, PI and angiogenesis influence LN status and survival time of CMC-bearing female dogs. Our hypotheses were that metastases are more frequent in LN that drain high grade, highly proliferative, VI (+) mammary carcinomas, and that survival time is shorter in female dogs bearing CMC of those characteristics.

Materials and methods

A total of 136 mammary carcinomas were analysed. The tumours selected were divided into two groups. Group I consisted of file cases (historical analysis), and group II consisted of clinical cases (prospective analysis). File cases consisted of all spontaneous invasive non-inflammatory mammary carcinomas of female dogs submitted with regional lymph nodes (LN) to the Institute of Pathology of the School of Veterinary Sciences of the University of La Plata (Buenos Aires, Argentina) during 23 years ($n = 102$).

Clinical cases consisted of invasive mammary carcinomas of female dogs admitted at the Small Animals Clinics of the School of Veterinary Sciences of the University of La Plata from 2004 to 2008 ($n = 34$), receiving surgical treatment only, and available for periodic clinical monitoring. Bitches with concurrent diseases that could limit their survival time were excluded from the study. Tumour size was assessed using a slide calliper during gross examination of the resected specimens. In approximately spherical tumours, the diameter was documented; otherwise, the major diameter was registered. Histopathological and immunohistochemical procedures were applied to the tumours of both groups ($n = 136$) to establish their HG, VI, PI, MVD and VEGFR2 expression. Lymph node (LN) status was recorded.

Histopathology

Tumours were classified as HG I, II or III according to the Elston & Ellis score, HG I being the most differentiated. The score was constructed based on tubule formation, nuclear pleomorphism and mitotic count (for a field diameter of 0.47 mm of the Nikon® Eclipse 50*i* microscope, Nikon Inc., Melville, NY, USA). Presence of neoplastic emboli in peri- or intra-tumour vessels was recorded as vascular invasion positive VI (+). LN status was consigned as (+) or (−) regarding the finding of metastases from mammary carcinoma. Negative LN were reprocessed. For that purpose, four new sections were obtained from the original blocks, separated by 100 μm each. Three of them (sections 1, 3 and 4) were routinely stained and the remaining one immunolabelled (see below).

Immunohistochemistry

Sections (5 μm thick) from the selected tumours were dewaxed in xylol, rehydrated through descending graded alcohols and heat-treated in 10 mM citrate buffer for antigen retrieval. To block nonspecific binding, a milk/phosphate-buffered saline (PBS) solution was applied to each slide (commercial skimmed milk powder, 4% in PBS) for 20 min. LSAB-2 was used both as the detection and as the revealing system (LSAB-2 System, HRP, K0673, DAKO Co., Dakocytomation, Carpinteria, CA, USA). Tissue sections were dehydrated in

graded ethanol, cleared in xylene and coverslipped after counterstaining with haematoxylin. At least one section was used as a negative control by omitting the primary antibody. Reproccessed LN were immunostained using the mouse anti-AE1 – AE3 cytokeratin antibodies (AE1/AE3, N1590, Dakocytomation) in search of micrometastases. LN with micrometastases were consigned as LN (+). The PI of the neoplastic cells was estimated by anti-PCNA antibody diluted 1/50 in PBS (anti-PCNA, clone PC10, M0879, DAKO Co.). Antigen retrieval was performed by immersion of the slides in polypropylene Coplin staining jars containing citrate buffer (pH 6.0, 10 mM). The jars were then placed in the steam bowl of an electric steamer for 40 min after boiling the water in the base unit. About 1000 cells per specimen were counted with ×400 magnification, and classified as positive (labelled) or negative (non-labelled). Cells showing nuclear golden brown labelling, regardless of its intensity, were considered positive. Positive cells undergoing mitosis were also included. Two pathologists of our group (JRI and MED) blindly analysed the first 20 specimens to set the criteria. For the quantification of PCNA expression, the PI was obtained as follows: PI = positive cells/1000 cells.

Microvessels were immunohistochemically labelled for anti-von Willebrand factor (vWf, clone F8/86 M 0616, DAKO Co., Dakocytomation) diluted 1/50 in PBS to estimate MVD. Antigens were unmasked using a microwave oven. Slides were placed in microwave-safe Coplin jars containing citrate buffer (pH 6.0, 10 mM for two cycles of 4 min at 750 W). Twenty high-power fields per case were analysed. Isolated, clustered cells or cord structures as well as vessels with a recognisable lumen were recorded as positive elements and expressed as elements/field. Taking this datum into account and the 0.075 mm^2 observation field surface, the results were finally expressed as elements/mm^2. VEGFR2 was detected using mouse anti-Flk-1 antibody diluted 1/100 in PBS (Flk-1-A/3-: sc-6251, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Immunoreactivity was enhanced with a microwave oven. Flk-1 labelling was quantified using an image analysis computer software (ImagePro Plus, v6.3, Media Cybernetics, Rockville, MD, USA). For that purpose, optical density parameters were measured in 20 fields from each sample using a 40× objective. A digital camera (EvolutionVF, QImaging, Surrey, Canada) mounted on a microscope (Olympus BX50, Olympus, Tokyo, Japan) was used to capture images with a resolution of 640 × 480 px, RGB and tagged image file format, with a relationship of 0.32 µm/px. Several optical parameters were measured and the integrated optical density (Flk-1 IOD) was chosen for statistical analyses.

The researchers performing the histopathological and immunohistochemical analyses were blinded to the clinical outcome of the animals.

Follow-up programme

The overall survival time after the histopathological diagnosis was 18 months, a time roughly equivalent to the human 5-year survival period. Physical and diagnostic imaging studies were performed every 6 months during the follow-up period. To state the cause of death, complete necropsy was performed when possible. When the body was not available for necropsy, clinical signs and the finding of radiographical images consistent with metastasis allowed us to classify the event as a tumour-related death. Animals whose cause of death remained uncertain were censored from the analysis. Once the follow-up period ended, the animal patients were classified according to their survival time as: low survival – (LS; dead before 6 months post-histopathological diagnosis), medium survival – (MS; dead between 6 and 18 months post-histopathological diagnosis) and high survival animals – (HS; alive at the end of the study). Death from a cause other than metastases from mammary tumours, and patients unavailable for follow-up or beginning of chemotherapy were considered censoring events.

Statistical analyses

Descriptive statistics, as well as Student's *t*, ANOVA, chi-square and correlation tests were performed by means of Microsoft® Excel 2002 software (Buenos Aires, Argentina). Student's *t*-test was performed to compare mean values of PI, MVD, Flk-1/IOD between tumours with different LN status, VI status and survival time. Differences between mean

values of PI, MVD, Flk-1/IOD of HG I, II and III tumours were analysed by two-way ANOVA. Chi-square test with Yates correction was used for categorical variables. Pearson and Spearman correlation coefficients were computed to assess the relation between PI and MVD and Flk-1/IOD, and between MVD and Flk-1/IOD. A multiple logistic regression model was applied to assess parameters independently associated with LN status. Survival time curves were generated by the Kaplan–Meier product limit method, using SPSS 10.0.1 for Windows (Statistical Product and Service Solutions). A log-rank test was used to compare the curves. The α-level was fixed at 0.05.

Results

Brownish nuclear labelling was observed in anti-PCNA-treated specimens. Both microvessels and some cell clusters and cords were positive to anti-vWf (Fig. 1). Anti-Flk-1-stained tumours showed positive signal not only in endothelial cells, but also in many carcinoma cells. Labelling was mostly cytoplasmic (Fig. 2). Frequency distribution of HG, VI and LN status are shown in Table 1. LN (+) included the reprocessed LN showing micrometastases (10%). Mean PI was 42.2 (with a standard deviation of 9.85). MVD mean value was 84.9 ± 53.5, being 70 the median. Flk-1/IOD values showed a wide dispersion (mean 289.65 ± 264.14).

Table 2 shows the association between the different features studied. Briefly, tumours of HG I, II and III showed statistically significant differences regarding PI, Flk-1/IOD and MVD. Minor differences were found in VI between HG I and HG II tumours. A strong association was found between VI and PI, Flk-1/IOD and ST. MVD was quite different between tumours with/without VI ($P = 0.014$). However, those differences were not significant when analysed as a categorical variable (low MVD and high MVD, being the median the cut-off value). PI varied between tumours of different Flk-1/IOD in a MVD-independent fashion, but not between tumours of different MVD. Flk-1/IOD and MVD were significantly correlated to each other. All the features studied showed significant association with LN status when analysed by means of bivariate analyses. From multivariate analyses, only VI and Flk-1/IOD were selected to predict LN status.

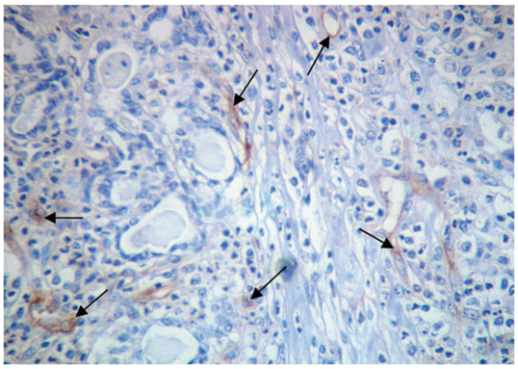

Figure 1. Canine mammary tumour. Arrows point to some endothelial, and isolated or clustered cells positive to anti-vWf antibody (Immunohistochemistry. Chromogen: diaminobenzidine; counterstain: haematoxylin). Objective ×20.

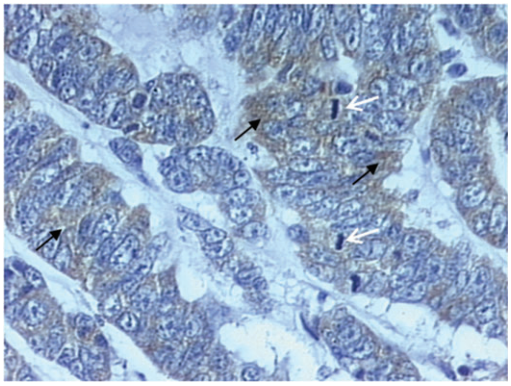

Figure 2. Canine mammary tumour. Many carcinoma cells show intense cytoplasmic reactivity to anti-Flk-1 (black arrows). White arrows point to mitotic figures (Immunohistochemistry. Chromogen: diaminobenzidine; counterstain: haematoxylin). Objective ×40.

Thirty-four animals fulfilled the inclusion criteria for group II. The group consisted of dogs from various breeds, ranging from 4 to 15 years old (mean value: 9.6). Tumours of most animals (31/34) were found in one of the three caudal pairs of glands. Size of the masses ranged from 0.3 to 15 cm. Sixteen out of 34 cases had LN (+). Eight animals were censored from the follow-up programme. Six of the animal patients had a low survival time, while one of them had a medium ST and the remaining 19 had a high ST. LN metastases were more frequent in >3 cm nodules than in smaller ones ($P = 0.028$). Size was related neither to angiogenic nor to

Table 1. Frequency of lymph node metastases and vascular invasion according to tumours' histological grade, over 136 cases

HG	*n*	%/t*n*	LN (+)	%/LN (+)	LN (−)	%/LN (−)	VI (+)	%/VI (+)	VI (−)	%/VI (−)
I	23	16.91	1	1.35	22	35.48	1	2.50	22	22.92
II	44	32.35	22	29.73	22	35.48	2	5.00	42	43.75
III	69	50.74	51	68.92	18	29.03	37	92.50	32	33.33
Total	136		74		62		40		96	

+, presence of metastases (or presence of emboli if it is VI+); −, absence of metastases/emboli; HG, histological grade; LN, lymph node; *n*, number of cases; t*n*, total *n*; VI, vascular invasion.

Table 2. Association between the studied features taken in pairs and statistical significance of the differences found

	VI					LN		ST	
HG	No	Yes	PI	MVD (elements/mm^2)	Flk-1/IOD	(+)	(−)	L/M	H
(I)	22	1	36.8	53	68725.1	1	22	1	6
(II)	42	2	39.3	75.5	198948.3	22	22	3	4
(III)	32	37	45.8	101.3	374792.4	51	18	3	9
	I–II NS I–III and II–III: $P < 0.001$[a]		$P = 0.0006$[b]	$P = 0.00021$[b]	$P = 0.0001$[b]	I–II $P = 0.0003$ I–III $P = 0.00008$ II–III $P = 0.005$[a]		NS[a]	
VI									
No			39.4	77.6	181113.6	38	58	3	16
Yes			48.7	102.1	470204.3	36	4	4	3
			$P = 0.00001$[c]	$P = 0.014$[c]	$P = 0.0001$[c]	$P = 0.0000008$[a]		$P = 0.0175$[a]	
PI									
				r: 0.058 NS	*r*: 0.329 $P = 0.01$	45.08 $P = 0.00013$[c]	38.7	41.25 NS[c]	40.7
MVD (elements/mm^2)									
					r: 0.270 $P = 0.01$	89.4 $P = 0.0009$[c]	68.47	63.59 NS[c]	66.7
Flk-1/IOD									
						440857.15 $P = 0.00001$[c]	57607.32	368911.9 NS[c]	216 231
LN									
(+)								6	7
(−)								1	12
								$P = 0.0167$[a]	

Flk-1/IOD, integrated optic density of Flk-1 signal; HG, histological grade; LN, lymph node status; MVD, microvessel density; NS, non-significant differences; PI, proliferation index; ST, survival time (L, low; M, medium; H, high); VI, vascular invasion.
[a]Chi square test.
[b]ANOVA test.
[c]Student's *t*-test.

proliferative activity. Data revealed that the probability of a CMC-bearing bitch to remain alive at 1, 4, 5 and 14–18 months was 0.91, 0.87, 0.81 and 0.77, respectively (Fig. 3). Besides LN status, VI was the only feature positively correlated with survival time (Table 2, Fig. 4).

Discussion

We found few published studies where the sample collection and methods (namely, histological grading) were equivalent to ours. This prevented us from objectively comparing the data obtained. Therefore, to enrich data comparison, results from studies comprising benign tumours are also discussed, provided that findings specific from malignant tumours were clearly stated. We also considered of interest to point out the results obtained by other researchers on FMC.

Our results on HG are compared here with those obtained by the same or similar assignment systems. Results on the association between PI and HG are consistent with that observed by other

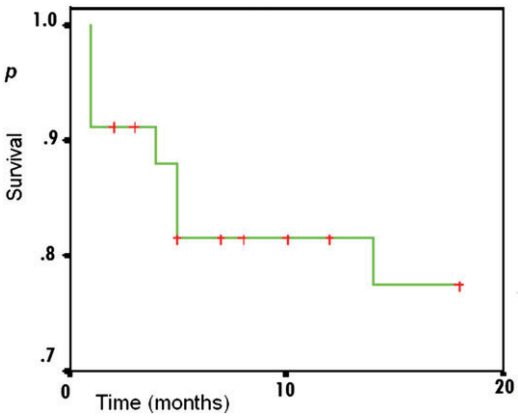

Figure 3. Overall survival. Kaplan–Meier survival curve for bitches in follow-up programme. *x*-axis: follow-up time in months; *y*-axis: probability of survival (*P*). The probability of a CMC-bearing bitch to remain alive at 1, 4, 5 and 14–18 months was 0.91, 0.87, 0.81 and 0.77, respectively.

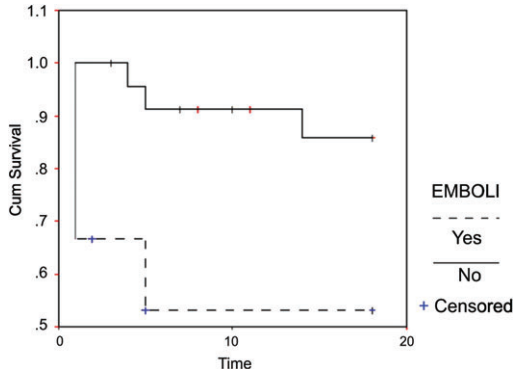

Figure 4. Survival functions for vascular invasion. Kaplan–Meier survival curves for tumours with or without vascular invasion (neoplastic emboli). *x*-axis: follow-up time in months; *y*-axis: probability of survival (*P*). Log rank = 5.65. For one degree of freedom $P = 0.0175$**.

authors in CMC and FMC. The HG of the tumours was related to the finding of VI and to LN status, in agreement with the study by Rasotto *et al.*[25] Seixas *et al.* found differences in VI but not in LN status among FMC of different HG.[21] Few previous reports, such as those by Millanta *et al.*, Al Dissi *et al.*, Restucci *et al.* and Santos *et al.*, have examined the association between HG and at least one of the angiogenic parameters analysed here (MVD and VEGFR2).[22,32,45,47,50] The former three were carried out in series of 26 to 40 carcinomas. Both determinations (MVD and VEGFR2) were performed only in Al Dissi's study.[32] In the present study, we observed an increase in VEGFR2 expression from HG I to HG III tumours. Restucci *et al.* found that less differentiated tumours also showed higher VEGFR2 expression, while Santos *et al.* did not find a significative association between HG and VEGFR2 expression.[47,50] In this study, and in agreement with that found by other authors, high-grade carcinomas also presented higher MVD. Conversely, Al Dissi *et al.* found no significant association between VEGFR or MVD and HG.[32] Although the association established here between high HG and MVD/VEGFR2 is not stated as a cause–effect relationship, HG may be useful to predict angiogenic behaviour of the CMC. Assignment of a HG to a tumour requires achieving some expertise and always implies subjectivity. Despite those disadvantages, it comes out as a useful tool as a prognostic indicator in CMC as higher HGs concur with higher angiogenic, proliferative, invasive and metastatic tumour phenotypes, as shown in this work. Besides, with appropriate training, it may be carried out in any pathology laboratory as it has low cost and no high-tech is required.

Concerning angiogenesis, we found that the two variables analysed were related to each other, in agreement with another study.[46] VEGF/VEGFR2 downstream signalling pathway triggers proliferation, migration and survival of endothelial cells, among other consequences. Thereby, a greater number of vessels per area could be expected. However, opposite results have been published by Al Dissi.[32] To our knowledge, there are two studies on LN status depending on MVD in veterinary medicine, which report results obtained from samples comprising few malignant tumours. Both groups of researchers noted a trend towards higher frequencies of positive LN in tumours with higher MVD.[66,67] In this report we demonstrated the association between VEGFR2 and LN status by means of univariate and multivariate analyses, opposite to the results reported by Santos *et al.*[50] Based on our results, tumour masses with higher MVD are more likely to grow and reach and invade those vessels, giving rise to neoplastic emboli. Conversely, in a study with 29 tumours, Millanta *et al.* found no significant differences in MVD between tumours with or without emboli.[22] In the present study, carcinomas exhibiting VI also showed higher

VEGFR2 expression. To the best of our knowledge, no other published work has dealt with the association between high expression of VEGFR2 and the finding of neoplastic emboli. The direct correlation found here between such features may be explained not only by the presence of a broader vascular bed available to be invaded but also by the abnormal structural traits and leakier neoformed vessels, as a result of the VEGF/VEGFR-2 signalling pathway. Besides the correlation between VI and tumour angiogenesis demonstrated, VI resulted an independent prognostic factor for LN status in the multivariate analysis performed, in agreement with that observed by Santos *et al.*[34]

Significant differences in PI were found between tumours with or without VI, in agreement with the results by Sarli *et al.*,[60] and also between tumours with different LN status. A high up-regulation in PCNA expression levels was demonstrated when comparing LN (+)/(−) tumours (ratio 2.26). The relationship between PI and HG was stated above. Regarding PI and angiogenesis, VEGFR2 high expressing populations showed higher PI, opposite to that found by Al Dissi *et al.*[32] Our results can be explained by the fact that VEGF/VEGFR2 binding induces proliferation of carcinoma cells (as well as of endothelial cells) by autocrine and paracrine pathways, both in human and canine tissues. In carcinoma cells, cytoplasmic immunolabelling of VEGFR2 was observed, in agreement with the results obtained by others. This finding might be explained by the fact that many proteins may be detected within the endomembranous system when they are not anchored to the plasma membrane yet. Several recent publications have demonstrated the importance of VEGFR-2 endocytosis in its biological function and signalling. A higher proliferative activity does not seem to rely on a greater number of vessels perfusing the tissue, as a higher MVD *per se* was not associated to a higher PI.

Overall survival time was assessed in a smaller sample than that used to estimate the other parameters discussed above. Among HG, PI, MVD, VEGFR2, VI and LN status, only VI and LN status were related to survival time. The LN status is known to be of prognostic value in dogs and cats.[1,3,5,17,27,34,39]

PI and VEGFR2 were the only variables close to a significant *P*-value ($P = 0.12$ and 0.15, respectively). An association between low PI of the tumour and higher survival time has been reported in bitches, as well as in queens.[21,31,51] Based on our results, differences in survival time among bitches cannot be related to HG ($P = 0.34$) or to MVD ($P = 0.8$), opposite to data reported in bitches and queens by others.[21,22,34,51−53] To our knowledge, in those studies of CMC which assessed the relationship between VI and survival time, VI was included as a part of a histological stage or even as a part of HG. Sarli *et al.* and Ito *et al.* found differences in survival time of animal patients bearing tumours classified as stage II, being that stage, defined by the authors, based on VI.[27,60] Others found that survival time of queens bearing mammary carcinomas was related to VI.[21]

The histological type of the tumour and LN status of the animal patient have been recorded in pathologists' reports for many decades. Other histological features, namely VI and HG, are increasingly included, sometimes as a part of the description and sometimes enriching the definitive classification of the mass. However, consensus is lacking on the usefulness of those features in the prognosis of canine and feline mammary carcinomas to justify their inclusion as a rule and become data requested by clinicians.[21,25,29,33] Our findings highlight the strong association of those histopathological features with the main prognostic factor, LN status, and even with survival time (VI).

In the event that LN is found negative by the histopathological routine method, searching for micrometastases by means of immunohistochemical techniques may reverse the diagnosis in many cases, as shown by our data, in agreement with previous studies.[12] If LN status diagnosis remains negative, or when the LN is missing, every feature showing association with LN status becomes increasingly outstanding for prognosis. Pathological specimens lacking axillary LN, which drain carcinomas arising from thoracic mammary glands, are rather usual. Although recommended, these carcinomas are seldom resected because surgical approach is hard to achieve.

It is worth pointing out that, besides conventional chemotherapeutic agents, anti-angiogenic

strategies increasingly used in humans may change the scene in veterinary oncology practice. The main anti-angiogenic strategy is tyrosine kinase inhibition by small molecules or monoclonal antibodies targeting specific RTK. Therefore, updating knowledge on signalling pathways involved in angiogenesis in canine tumours is imperative to profit by pharmacological progress. Our results strongly support the close association between VEGFR2 high expressing CMC and LN metastases, in samples of well over a hundred of CMC and statistically significant differences. We found a trend to shorter survival of animal patients bearing VEGFR2 high expressing CMC, being this an issue that remains to be further analysed.

Being aware that nearly half of the bitches with CMC will be cured with surgery alone, conclusions from research on prognostic factors should provide sufficient predictive information for the clinician, deciding in a case-by-case basis, to identify those animal patients which will benefit from chemotherapy or other therapeutic alternatives.

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Conflict of interests

The authors do not have any conflict of interests to declare.

References

1. Lana SE, Rutteman GR and Withrow SJ. In: *Withrow and MacEwen's Small Animal Clinical Oncology*. 4th edn., SJ Withrow and DM Vail Eds., St Louis, Saunders Elsevier, 2007: 619–636.
2. Sleeckx N, de Rooster H, Veldhuis Kroeze EJB, Van Ginneken C and Van Brantengem L. Canine mammary tumours, an overview. *Reproduction in Domestic Animals* 2011; **46**: 1112–1131. doi:10.1111/j.1439-0531.2011.01816.x.
3. Sorenmo K, Rasotto R, Zappulli V and Goldschmidt MH. Development, anatomy, histology, lymphatic drainage, clinical features, and cell differentiation markers of canine mammary gland neoplasms. *Veterinary Pathology* 2011; **48**: 85–97. doi:10.1177/0300985810389480.
4. Misdorp W. Tumors of the mammary gland. In: *Tumors in Domestic Animals*. 4th edn., DJ Meuten Ed., Ames, Iowa State University Press, 2002: 575–596, 764.
5. Misdorp W, Else RW, Hellmén E and Lipscomb TP. *Histological Classification of Mammary Tumors of the Dog and the Cat*. Vol VII. Washington, AFIP, 1999.
6. Sorenmo KU, Kristiansen VM, Cofone MA, Shofer FS, Breen AM, Langeland M, *et al.* Canine mammary gland tumours; a histological continuum from benign to malignant; clinical and histopathological evidence. *Veterinary and Comparative Oncology* 2009; **7**: 162–172. doi:10.1111/j.1476-5829.2009.00184.x.
7. Leber MF and Efferth T. Molecular principles of cancer invasion and metastasis (review). *International Journal of Oncology* 2009; **34**: 881–895. doi:10.3892/ijo_00000214.
8. London CA. Tyrosine kinase inhibitors in veterinary medicine. *Topics in Companion Animal Medicine* 2009; **24**: 106–112. doi:10.1053/j.tcam.2009.02.002.
9. Bavcar S and Argyle DJ. Receptor tyrosine kinase inhibitors: molecularly targeted drugs for veterinary cancer therapy. *Veterinary and Comparative Oncology* 2012; **10**: 163–173. doi:10.1111/j.1476-5829.2012.00342.x.
10. Klopfleisch R, Klose P, Weise C, Bondzio A, Multhaup G, Einspanier R, *et al.* Proteome of metastatic canine mammary carcinomas: similarities to and differences from human breast cancer. *Journal of Proteome Research* 2010; **9**: 6380–6391. doi:10.1021/pr100671c.
11. Klopfleisch R, von Euler H, Sarli G, Pinho SS, Gärtner F and Gruber AD. Molecular carcinogenesis of canine mammary tumors: news from and old disease. *Veterinary Pathology* 2011; **48**: 98–116. doi:10.1177/0300985810390826.
12. Matos AJ, Lopes CC, Faustino AM, Carvalheira JG, Dos Santos MS, Rutteman GR, *et al.* MIB-1 labelling indices according to clinico-pathological variables in canine mammary tumours: a multivariate study. *Anticancer Research* 2006; **26**: 1821–1826.
13. Matos AJF, Baptista CS, Gärtner MF and Rutteman GR. Prognostic studies of canine and feline mammary tumours: the need for standarized procedures. *The Veterinary Journal* 2012; **193**: 24–31. doi:10.1016/j.tvjl.2011.12.019.
14. Goldschmidt M, Peña L, Rasotto R and Zappulli V. Classification and grading of canine mamary tumors. *Veterinary Pathology* 2011; **48**: 117–131. doi:10.1177/0300985810393258.

15. Queiroga FL, Pires I, Parente M, Gregorio H and Lopes CS. COX-2 over-expression correlates with VEGF and tumour angiogenesis in canine mammary cancer. *The Veterinary Journal* 2011; **189**: 77–82. doi:10.1016/j.tvjl.2010.06.022.
16. Lamp O, Honscha KU, Schweizer S, Heckmann A, Blaschzik S and Einspanier A. The metastatic potential of canine mammary tumours can be assessed by mRNA expression analysis of connective tissue modulators. *Veterinary and Comparative Oncology* 2013; **11**: 70–85. doi:10.1111/j.1476-5829.2011.00303.x.
17. Szczubial M and Lopuszynski W. Prognostic value of regional lymph node status in canine mammary carcinomas. *Veterinary and Comparative Oncology* 2011; **9**: 296–303. doi:10.1111/j.1476-5829.2011.00268.x.
18. Jakab C, Halász J, Kiss A, Schaff Z, Szász AM, Rusvai M, *et al.* Evaluation of microvessel density (MVD) in canine mammary tumours by quantitative immunohistochemistry of the claudin-5 molecule. *Acta Veterinaria Hungarica* 2008; **56**: 495–510. doi:10.1556/AVet.56.2008.4.7.
19. Betz D, Schoenrock D, Mischke R, Baumgärtner W and Nolte I. Postoperative treatment outcome in canine mammary tumors. Multivariate analysis of the prognostic value of pre-and postoperatively available information. *Tierärztliche Praxis Kleintiere* 2012; **40**: 235–242.
20. Ferreira E, Bertagnolli AC, Cavalcanti MF, Schmitt FC and Cassali GD. The relationship between tumour size and expression of prognostic markers in benign and malignant canine mammary tumours. *Veterinary and Comparative Oncology* 2009; **7**: 230–235. doi:10.1111/j.1476-5829.2009.00193.x.
21. Seixas F, Palmeira C, Pires MA, Bento MJ and Lopes C. Grade is an independent prognostic factor for feline mammary carcinomas: a clinicopathological and survival analysis. *The Veterinary Journal* 2011; **187**: 65–71. doi:10.1016/j.tvjl.2009.10.030.
22. Millanta F, Silvestri G, Vaselli C, Citi S, Pisani G, Lorenzi D, *et al.* The role of vascular endothelial growth factor and its receptor Flk-1/KDR in promoting tumour angiogenesis in feline and canine mammary carcinomas: A preliminary study of autocrine and paracrine loops. *Research in Veterinary Science* 2006; **81**: 350–357. doi:10.1016/j.rvsc.2006.01.007.
23. Millanta F, Caneschi V, Ressel L, Citi S and Poli A. Expression of vascular endothelial growth factor in canine inflammatory and non-inflammatory mammary carcinoma. *Journal of Comparative Pathology* 2010; **142**: 36–42. doi:10.1016/j.jcpa.2009.06.004.
24. Sleeckx N, Van Brantengem L, Van den Eyden G, Fransen E, Casteleyn C, Cruchten Van, *et al.* Angiogenesis in canine mammary tumours: a morphometric and prognostic study. *Journal of Comparative Pathology* 2014; **150**: 175–183. doi:10.1016/j.jcpa.2013.09.005.
25. Rasotto R, Zappulli V, Castagnaro M and Goldschmidt MH. A retrospective study of those histopathologic parameters predictive of invasion of the lymphatic system by canine mammary carcinomas. *Veterinary Pathology* 2012; **49**: 330–340. doi:10.1177/0300985811409253.
26. Ressel L, Puleio R, Loria GR, Vannozi I, Millanta F, Caracappa S, *et al.* HER-2 expression in canine morphologically normal, hyperplastic and neoplastic mammary tissues and its correlation with the clinical outcome. *Research in Veterinary Science* 2013; **94**: 299–305. doi:10.1016/j.rvsc.2012.09.016.
27. Itoh T, Uchida K, Ishikawa K, Kushima K, Kushima E, Tamada H, *et al.* Clinicopathological survey of 101 canine mammary gland tumors: differences between small-breed dogs and others. *The Journal of Veterinary Medical Science* 2005; **67**: 345–347.
28. Martín de las Mulas J, Millán Y and Dios R. A prospective analysis of immunohistochemically determined estrogen receptor alpha and progesterone receptor expression and host and tumor factors as predictors of disease-free period in mammary tumors of the dog. *Veterinary Pathology* 2005; **42**: 200–212. doi:10.1354/vp.42-2-200.
29. Peña L, De Andrés PJ, Clemente M, Cuesta P and Pérez-Alenza MD. 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* 2013; **50**: 94–105. doi:10.1177/0300985812447830.
30. Clemente M, Pérez-Alenza MD, Illera JC and Peña L. Histological, immunohistological, and ultraestructural description of vasculogenic mimicry in canine mammary cancer. *Veterinary Pathology* 2010; **47**: 265–274. doi:10.1177/0300985809353167.
31. Preziosi R, Sarli G, Benazzi C, Mandrioli L and Marcato PS. Multiparametric survival analysis of histological stage and proliferative activity in feline mammary carcinomas. *Research in Veterinary Science* 2002; **73**: 53–60. doi:10.1016/S0034-5288(02)00042-5.
32. Al-Dissi AN, Haines DM, Singh B and Kidney BA. Immunohistochemical expression of vascular endothelial growth factor and vascular endothelial growth factor receptor-2 in canine simple

mammary gland adenocarcinomas. *Canadian Veterinary Journal* 2010; **51**: 1109–1114.
33. Santos AA, Lopes CC, Ribeiro JR, Martins LR, Santos JC, Amorim IF, *et al.* Identification of prognostic factors in canine mammary malignant tumours: a multivariable survival study. *BMC Veterinary Research* 2013; **9**: 1–11. doi:10.1186/1746-6148-9-1.
34. Santos AA, Oliveira JT, Lopes CC, Amorim IF, Vicente CM, Gärtner F, *et al.* Immunohistochemical expression of vascular endothelial growth factor in canine mammary tumours. *Journal of Comparative Pathology* 2010; **143**: 268–275. doi:10.1016/j.jcpa.2010.04.006.
35. Winston J, Craft DM, Scase TJ and Bergman PJ. Immunohistochemical detection of HER-2/neu expression in spontaneous feline mammary tumours. *Veterinary and Comparative Oncology* 2005; **3**: 8–15. doi:10.1111/j.1476-5810.2005.00063.x.
36. Port Louis LR, Varshney KC and Nair MG. An immunohistochemical study on the expression of sex steroid receptors in canine mammary tumors. *International Scholarly Research Network Veterinary Science* 2012; **2012**: 378607. doi:10.5402/2012/378607.
37. Kato T, Kameoka S, Kimura T, Soga N, Abe Y, Nishikawa T, *et al.* Angiogenesis as a predictor of long-term survival for 377 Japanese patients with breast cancer. *Breast Cancer Research and Treatment* 2001; **70**: 65–74.
38. Uzzan B, Nicolas P, Cucherat M and Perret GY. Microvessel density as a prognostic factor in women with breast cancer: a systematic review of the literature and meta-analysis. *Cancer Research* 2004; **64**: 2941–2955.
39. Chang S, Chang C, Chang T and Wong M. Prognostic factors associated with survival two years after surgery in dogs with malignant mammary tumors: 79 cases (1998–2002). *Journal of the American Veterinary Medicine Association* 2005; **227**: 1625–1629.
40. Nieto A, Peña L, Pérez-Alenza MD, Sánchez MA, Flores JM and Castaño M. Immunohistologic detection of estrogen receptor alpha in canine mammary tumors: clinical and pathologic associations and prognostic significance. *Veterinary Pathology* 2000; **37**: 239–247.
41. Karayannopoulou M, Kaldrymidou E, Constantinidis TC and Dessiris A. Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method. *Journal of Comparative Pathology* 2005; **133**: 246–252. doi:10.1016/j.jcpa.2005.05.003.
42. Philibert JC, Snyder PW, Glickman N, Glickman LT, Knapp DW and Waters D. Influence of host factors on survival in dogs with malignant mammary gland tumors. *Journal of Veterinary Internal Medicine* 2003; **17**: 102–106.
43. Pérez Alenza M, Peña L, Nieto A and Castaño M. Clinical and pathological prognostic factors in canine mammary tumours. *Annali dell'Istituto Superiore di Sanità* 1997; **33**: 581–585.
44. Peña L, Nieto A, Pérez-Alenza D, Cuesta P and Castaño M. Immunohistochemical detection of Ki-67 and PCNA in canine mammary tumors: relationship to clinical and pathologic variables. *Journal of Veterinary Diagnostic Investigation* 1998; **10**: 237–246. doi:10.1177/104063879801000303.
45. Restucci B, de Vico G and Maiolino P. Evaluation of angiogenesis in canine mammary tumors by quantitative platelet endothelial cell adhesion molecule immunohistochemistry. *Veterinary Pathology* 2000; **37**: 297. doi:10.1354/vp.37-4-297.
46. Restucci B, Papparella S, Maiolino P and De Vico G. Expression of vascular endotelial growth factor in canine mammary tumors. *Veterinary Pathology* 2002; **39**: 488–493. doi:10.1354/vp.39-4-488.
47. Restucci B, Borzacchiello G, Maiolino P, Martano M, Paciello O and Papparella S. Expression of vascular endothelial growth factor receptor Flk-1 in canine mammary tumours. *Journal of Comparative Pathology* 2004; **130**: 99–104. doi:10.1016/j.jcpa.2003.07.001.
48. Lagadic M, Estrada M, Camadro JP, Durand P and Goebel J. Tumeurs mammaires de la Chienne: critères du pronostic histologique et intérêt d'un grading. *Recueil de Médicine Vétérinaire Spécial Cancérologie* 1990; **166**: 1035–1042.
49. Im KS, Kim NH, Lim HY, Kim HW, Shin JI and Sur JH. Analysis of a new histological and molecular-based classification of canine mammary neoplasia. *Veterinary Pathology* 2014; **51**: 549–559. doi:10.1177/0300985813498780.
50. Santos A, Lopes C, Gärtner F and Matos AJF. VEGFR-2 expression in malignant tumours of the canine mammary gland: a prospective survival study. *Veterinary and Comparative Oncology* 2014. doi:10.1111/vco.12107.
51. Castagnaro M, Casalone C, Bozzetta E, De Maria R, Biolatti B and Caramelli M. Tumour grading and the one-year post-surgical prognosis in feline mammary carcinomas. *Journal of Comparative Pathology* 1998; **119**: 263–275.
52. Millanta F, Lazzeri G, Vannozzi I, Viacava P and Poli A. Correlation of vascular endothelial growth factor expression to overall survival in feline invasive mammary carcinomas. *Veterinary*

Pathology 2002; **39**: 690 – 696. doi:10.1354/vp.39-6-690.
53. Mills SW, Musil KM, Davies JL, Hendrick S, Duncan C, Jackson ML, *et al.* Prognostic value of histologic grading for feline mammary carcinoma: a retrospective survival analysis. *Veterinary Pathology* 2015; **52**: 238 – 249. doi:10.1177/0300985814543198.
54. Pérez Alenza M, Peña L, del Castillo N and Nieto A. Factors influencing the incidence and prognosis of canine mammary tumours. *Journal of Small Animal Practice* 2000; **41**: 287 – 291.
55. Elston CW and Ellis LO. 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* 1991; **19**: 403 – 410.
56. Maga G and Hübscher U. Proliferating cell nuclear antigen (PCNA): a dancer withmany partners. *Journal of Cell Science* 2003; **116**: 3051 – 3060. doi:10.1242/jcs.00653.
57. Stuart-Harris R, Caldas C, Pinder SE and Pharoah P. Proliferation markers and survival in early breast cancer: A systematic review and metaanalysis of 85 studies in 32,825 patients. *The Breast* 2008; **17**: 323 – 334. doi:10.1016/j.breast.2008.02.002.
58. Funakoshi Y, Nakayama H, Uetsuka K, Nishimura R, Sasaki N and Doi K. Cellular proliferative and telomerase activity in canine mammary gland tumors. *Veterinary Pathology* 2000; **37**: 177 – 183.
59. Kumaraguruparan R, Prathiba D and Nagini S. Of humans and canines: immunohistochemical analysis of PCNA, Bcl-2, p53, cytokeratin and ER in mammary tumours. *Research in Veterinary Science* 2006; **81**: 218 – 224.
60. Sarli G, Preziosi R, Benazzi C, Castellani G and Marcato PS. Prognostic value of histologic stage and proliferative activity in canine malignant mammary tumors. *Journal of Veterinary Diagnostic Investigation* 2002; **14**: 25 – 34. doi:10.1177/104063870201400106.
61. Schneider BP and Miller KD. Angiogenesis of breast cancer. *Journal of Clinical Oncology* 2005; **23**: 1782 – 1790.
62. Rapisarda A and Melillo G. Role of the VEGF/VEGFR axis in cancer biology and therapy. *Advances in Cancer Research* 2012; **114**: 237 – 267. doi:10.1016/B978-0-12-386503-8.00006-5.
63. Ferrara N, Gerber HP and LeCouter J. The biology of VEGF and its receptors. *Nature Medicine* 2003; **9**: 669 – 676.
64. Schoeffner DJ, Matheny SL, Akahane T, Factor V, Berry A, Merlino G, *et al.* VEGF contributes to mammary tumor growth in transgenic mice through paracrine and autocrine mechanisms. *Laboratory Investigation* 2005; **85**: 608 – 623.
65. Perrot-Applanat M and Di Benedetto M. Autocrine functions of VEGF in breast tumor cells: adhesion, survival, migration and invasion. *Cell Adhesion & Migration* 2012; **6**: 547 – 553. doi:10.4161/cam.23332.
66. Griffey SM, Verstraete FJM, Kraegel SA, Lucroy MD and Madewell BR. Computer-assisted image analysis of intratumoral vessel density in mammary tumors from dogs. *American Journal of Veterinary Research* 1998; **59**: 1238 – 1248.
67. Graham JC and Myers RK. The prognostic significance of angiogenesis in canine mammary tumors. *Journal of Veterinary Internal Medicine* 1999; **13**: 416 – 418.
68. Carvalho MI, Guimarães MJ, Pires I, Prada J, Silva-Carvalho R, Lopes C, *et al.* EGFR and microvessel density in canine malignant mammary tumours. *Research in Veterinary Science* 2013; **95**: 1094 – 1099. doi:10.1016/j.rvsc.2013.09.003.

