Aspects regarding intratumor angiogenesis and vasculogenic mimicry in canine mammary tumors

Aspecte privind angiogeneza intratumorală şi vasculogeneza imitată în tumorile mamare canine

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Abstract

Canine mammary tumors represent an important cause of morbidity and mortality in female dogs worldwide having a three times higher frequency than breast cancer in women. Neo-angiogenesis is critical for tumor growth, progression and metastases. The purpose of this study was to investigate neo-angiogenesis in canine mammary tumors and its possible utility as a prognostic biomarker by comparison with classic prognostic factors. In addition, we investigated if vasculogenic mimicry phenomenon of intratumor angiogenesis exists in this tumor type. Paraffin-embedded tissue samples from 21 female dogs with mammary cancers were immunostained for CD31 and Ki-67 nuclear proliferation marker, using Dako LSAB (Labelled streptavidin biotin)+System-HRP (Horseradish Peroxidase). Vasculogenic mimicry pattern was detected where necessary by double staining procedure. Statistical analysis was performed in Statistica software, $p<0.05$. We obtained a progressive increase of IMD (intratumor microvessel density) in poorly differentiated mammary carcinomas with high proliferative rate, comparing with differentiated ones ($p<0.0001$). The occurrence of blood channels was higher in vicinity of intratumor necrotic areas. Vasculogenic mimicry was observed more often in tumors with reduced stroma and numerous cancerous cells (such as solid mammary carcinomas), and in poorly differentiated canine mammary cancer. There was a statistical correlation between VM (vasculogenic mimicry) incidence and tumoral grade ($p<0.0001$). An increased incidence of VM was noticed in canine mammary carcinomas with high rate of intratumoral neo-angiogenesis showed by elevated IMD values ($p<0.0007$). Evaluation of neo-angiogenesis represents an additional factor that promotes the aggressiveness of canine mammary tumors, allowing the selection of a group of tumors with an aggressive behavior that generate vascular channels which facilitate tumor perfusion.

Keywords: *angiogenesis, immunohistochemistry, PAS reaction, vasculogenic mimicry.*

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Rezumat

Tumorile mamare canine reprezintă o cauză importantă a mortalității la cățea, având o incidență de trei ori mai mare decât cancerul de sân la femeie. Neo-angiogeneza este esențială pentru creşterea, dezvoltarea şi metatazarea tumorală. Scopul lucrării este de a investiga neo-angiogeneza în tumorile mamare canine, precum şi utilitatea prognostică a acestui biomarker prin compararea acestuia cu markeri clasici de apreciere a malignității. Actualul studiu urmăreşte de asemenea dacă fenomenul de "vasculogeneză imitată" poate fi întâlnit în acest tip de cancer. Probele tisulare provenite din tumori mamare de la nouă cățele, au fost înglobate în parafină şi ulterior imunomarcate pentru cuantificarea markerului endotelial CD31 şi a markerului de proliferare nuclear Ki-67, utilizându-se tehnica Dako LSAB (Labelled streptavidin biotin)+System-HRP (Horseradish Peroxidase). Fenomenul de "vasculogenic mimicry" a fost pus în evidență printr-o dublă colorare. Analiza statistică s-a făcut utilizând softul Statistica, considerându-se valori seminificative $p<0.05$. Rezultatele obținute au relevat o creştere progresivă a IMD în carcinoamele mamare slab diferențiate, cu rata mare de proliferare, comparativ cu cele diferentiate ($p<0.05$). Incidența canalelor de curgere sanguină a fost mai crescută în vecinătatea zonelor de necroză intratumorală. Fenomenul de "vasculogenic mimicry" a fost observat mai frecvent în tumorile cu stromă discretă şi cu un număr crescut de celule canceroase (cum ar fi carcinoamele solide), precum şi în carcinoamele mamare slab diferențiate. Studiul nostru a observat o corelație directă între incidența VM şi gradul tumoral ($p<0.0008$). Aspectul de VM a fost mai frecvent întâlnit în carcinoamele mamare cu o rată crescută a neo-angiogenezei intratumorale, indicată de valori crescute ale IMD ($p<0.04$). Cuantificarea neo-angiogenezei intratumorale reprezintă un factor adițional de apreciere a malignității tumorilor mamare canine, angiogeneza tumorală crescută facilitând în cazul tumorilor agresive generarea unor canele de curgere sanguină care să mențină perfuzia tumorii.

Cuvinte cheie: *angiogeneză, imunohistochimie, reacție PAS, vasculogeneză imitată.*

Introduction

Canine mammary cancer is the most common tumor in female dogs worldwide. Lymph node metastases, tumor size, ulceration, histological type and grade are some of the most important prognostic factors for mammary cancer patients (1). Recently, quantification of intratumor angiogenesis has been identified as a potentially useful prognosis factor for breast cancer. Previous reports have shown that a high intratumor microvessel density was associated with a high tendency of tumor cells dissemination, quick tumor development and reduced survivor period (2, 3). Using histopathological staining methods for blood vessel endothelial markers, a significant direct correlation was found between the highest microvessel density in histological sections of human invasive breast cancer and the occurrence of metastases. These findings notwithstanding, no studies have shown that angiogenesis is correlated with regional lymph node metastasis in breast cancer (4).

Having many similarities between canine mammary cancer and breast cancer in women, we consider that all the studies in this sense could deliver useful data for both women and female dogs regarding mammary cancer. In the present study we investigated the angiogenic profile of different types of canine mammary tumors by quantification of some specific parameters, such as intratumor microvessel density and microvessels' area. The prognostic value of intratumor angiogenesis was evaluated by comparison with classic prognostic factors. Furthermore, we investigated the incidence of vasculogenic mimicry pattern of intratumor angiogenesis and established some correlations with the prognosis (1, 2).

Material and methods

We performed a study on 21 female dogs diagnosed with mammary tumors, the cases being selected in a period of 11 months (October 2009 – August 2010). Improper tumors were avoided (poor formalin fixation, large necrotic intratumor areas). Tumor structures were provided by cadavers, or were represented by tumor biopsies received by the Pathology, Necropsy and Forensic

Medicine Department (University of Agricultural Science and Veterinary Medicine – USAMV - Cluj-Napoca, Romania). Canine mammary tumors originated from different breeds, such as: Cocker (5 subjects), German Shepherd (3 subjects), Stray dog (3 subjects), Teckel (2 subjects), Mioritic Romanian Shepherd (2 subjects), Boxer (2 subjects), Amstaff (1 subject), Irish Setter (1 subject), Doberman (1 subject) and Dalmatian (1 subject). The age of the female dogs varied from 2 to 13 years. The gross features of the tumors that have been included in the study provided useful data concerning malignancy and invasiveness (tumor size, ulceration, status of the lymph nodes).

Paraffin-embedded mammary tissues were processed at the time of diagnosis in the Pathology Department of USAMV Cluj-Napoca and immunohistochemical examinations (IHC) were performed. The study was approved by the local Ethics Committee.

Histology

Samples of tumor tissue were immersed in buffered 10% formalin, and then processed by paraffin technique (embedded in paraffin and sectioned using the microtome). Slides were stained following usual techniques, respectively Masson's tri-chromic and hematoxylin-eosin. The type of the mammary tumor was established according to WHO classification for mammary tumors and graded into three types (from grade I-less aggressive, to grade III-high aggressivity) (3). In order to grade the tumors we have evaluated the following parameters: nuclear grade, mitotic index, tubule and gland formation (1). For accuracy a malignancy marker was chosen, such as the Ki-67 nuclear antigen. Its utility in canine mammary cancer was proven by several studies, as Ki-67 correlates with the histological grade (1, 3).

Immunohistochemistry

Detection and monitoring of intratumor neo-angiogenesis and especially of the vasculogenic mimicry pattern were evaluated using immunohistochemical LSAB reaction, or dual staining respectively immunohistochemical and PAS reactions (where necessary). To identify neo-angiogenesis, tissue sections were immunostained for CD31 biomarker (DAKO monoclonal antibody, clone JC70A, IgG1 kappa isotype). Histological slides were fixed on silanized slides (DAKO) for 24 hours in 37°C, followed by deparaffination in xylene. Antigen retrieval was performed using a pressurized cooker and citrate solution (DAKO, citrate solution pH 6.1, code S1699). Endogenous peroxidase was inactivated by peroxidase blocking reagent (DAKO, Peroxidase and PA blocking reagent 3%, code S2003) for 5 minutes at room temperature. Primary monoclonal antibodies (anti-CD31) were maintained overnight, during 18 hours at 4°C, using a dilution of 1:30 (DAKO antibody diluents, code CES0809). The visualization of immunological reaction was performed using Universal LSABTM+kits (DAKO, CE-K0679 HRP rabbit/mouse/goat, code K0679); the counterstaining was performed with Mayer hematoxylin. Negative control was used in order to evaluate antibody specificity (the primary antibody was replaced with antibody diluent).

The same protocol was used for Ki-67 nuclear antigen (DAKO, clone KI-S5, IgG1 kappa isotype), the only difference being the dilution of primary monoclonal antibody (dilution 1:75).

Immunohistochemistry and PAS reaction

The present study is intended to examine canine mammary tumors by means of CD31 endothelial marker periodic acid–Schiff (PAS) dual staining to see if vasculogenic mimicry exists in these tumors. Before staining with Mayer's hematoxylin after the immunohistochemical reaction, the slides were immersed in periodic acid (aqueous solution 0.5%) and Schiff reactive (30 minutes) (PAS solutions prepared in Histology Department of USAMV Cluj-Napoca). Slides were rinsed with tap water and counterstained with Mayer's hematoxylin.

Table 1. Clinicopathological features of female dogs with mammary tumors based on classical prognostic factors

Variable	Percentage of cases [%]
Age	
<9 years	28.5
>9 years	71.5
Pregnancy	
Only one or none	71.4
Several	28.6
Castration	
Yes	14.3
No	85.7
Tumor size	
<5 cm	42.8
>5 cm	57.2
Lymph node invasion	
With invasion	33.3
Without invasion	66.7
Histological type	
Simple adenoma	19.1
Fibroadenoma	4.7
Solid simple carcinoma	19.1
Simple tubulopapillary carcinoma	19.1
Simple cystic carcinoma	9.4
Carcinoma in benign mixed tumor (BMT)	14.3
Complex carcinoma	14.3
Tumor type (or malignancy grade)	
Benign tumor	23.8
Malignant G I (well-differentiated)	19.1
Malignant G II (moderately differentiated)	42.8
Malignant G III (undifferentiated)	14.3

Neo-angiogenesis and vasculogenic mimicry evaluation; Ki-67 quantification

To evaluate the micro-vessel number, area and perimeter, we used a semiautomatic computerized analysis technique (Olympus Soft imaging solutions Cell B). CD31 was assessed in five representative microscopic fields in every tumor, magnified by 400x (*Figure 2*). Micro-vessels were counted in the area with the highest density (”hot spot”), after identification with a smaller magnification. Any isolated but immunohistochemically labeled endothelial cell (vessels without lumen) was quantified as a distinct micro-vessel. The microscopic images were obtained by Olympus BX51 microscope, connected to a photo digital camera (Olympus DP-25). Total microvascular area (TMA - expressed in μm^2 and percentage; average value obtained by monitoring micro-vessel area in 5 microscopic fields magnified of 400x), total microvascular perimeter (TMP - expressed in μm; average value obtained by monitoring micro-vessel’s perimeter in 5 microscopic fields magnified of 400x), average intratumor microvessel density (IMD resulted by counting the micro-vessels in 5 microscopic fields magnified of 400x), average micro-vessel area (MA obtained by formula TMA/IMD), and average microvascular perimeter (MP obtained by formula TMP/IMD) were related to microscopic image area (35442.98 μm^2).

The occurrence of vasculogenic mimicry pattern (PAS positive blood channels) was evaluated as follows: relatively frequently encountered (++), rarely met but present (+), and absent (-). VM was assessed in ten representative microscopic fields in every tumor, magnified by 400x. We monitored blood channels in the areas with the highest micro-vessel density (”hot spot”), after the identification with a smaller magnification.

Quantification of Ki-67 nuclear antigen was made by establishing the percentage of immunomarked cell (cells with brown nuclei) from all tumor cells/microscopic field. The count was achieved using two microscopic images magnified by 400x for every tumor, and 500-1000 tumor cells were counted per tumor. Ki-67 positive cells were counted in the area with the highest density, after identification with a smaller magnification. We used a semiautomatic computerized analysis technique (Olympus Soft imaging solutions Cell B).

Table 2. Markers used to assess malignancy in canine mammary tumors

Case nr.	Histopathologic diagnosis	HG	Tumor neo-angiogenesis						Ki-67 average value (%)
			IMD	TMA (µm2)	TMP (µm)	TMA (%)	Average MA	Average MP	
1.	Simple solid carcinoma	2	20.2	2824.21	1063.3	7.9	142.64	53.70	22.6
2.	Tubulopapillary carcinoma in BMT	1	8.4	1603.19	531.63	4.50	190.86	63.29	13.4
3.	Simple adenoma	-	6.6	3280.07	677.98	9.25	496.98	102.72	-
4.	Simple cystic carcinoma	1	11.8	2928.77	992.69	8.2	248.20	84.13	22.1
5.	Simple solid carcinoma	3	15.8	1527.17	679.11	4.30	96.66	42.98	26.7
6.	Simple tubulopapillary carcinoma	2	14	1923.58	721.71	5.42	137.4	51.55	22.4
7.	Solid anaplastic carcinoma	3	32.2	7136.85	2098.84	20.1	221.64	65.18	55.5
8.	Carcinoma in BMT	2	19.4	3510.2	1245.64	9.9	180.94	64.21	45.0
9.	Fibroadenoma	-	5.0	1853.86	465.99	5.20	370.77	93.20	-
10.	Simple adenoma	-	5.2	1605.86	2698.72	4.53	167.21	66.25	-
11.	Simple tubulopapillary carcinoma	1	12.7	2686.25	887.9	7.57	211.28	69.83	22.1
12.	Simple tubulary carcinoma	1	4.4	487.16	232.43	1.37	110.72	52.83	4.6
13.	Complex tubulopapillary carcinoma	2	10.4	1744.52	661.82	4.92	167.28	63.46	12.7
14.	Complex tubulopapillary carcinoma	2	10.1	1276.53	582.86	3.60	127.65	58.29	8.7
15.	Simple adenoma	-	5.5	2662.72	489.89	7.51	503.75	92.68	-
16.	Simple tubulopapillary carcinoma	2	11.1	1979.28	707.15	5.58	177.25	63.33	10
17.	Cystic carcinoma in BMT	2	15.5	2237.74	898.14	6.31	144.37	57.94	11.3
18.	Simple cystic carcinoma	2	15.6	1537.16	726.88	4.33	98.54	46.59	23.5
19.	Simple solid carcinoma	3	11.1	982.85	515.56	2.77	88.02	46.17	40.7
20.	Complex carcinoma	2	11.8	1139.24	605.86	3.21	96.55	51.34	26
21.	Simple adenoma	-	4.6	4526.79	925.84	12.77	536.19	111.38	-

HG: histological grade (G I - well-differentiated. G II - moderately differentiated. G III - undifferentiated).
MI: mitotic index.
IMD: intratumor microvessel density (microvessel number)/microscopic image aria.
TMA (µm2): total micro-vessel area /microscopic image area - average value obtained by monitoring micro-vessel area in 5 microscopic fields magnified by 400x.
TMP (µm): total micro-vessel perimeter/microscopic image area - average value obtained by monitoring micro-vessel perimeter in 5 microscopic fields magnified by 400x.
TMA (%): percentage value of TMA out of microscopic image area that was 35442.98 μm^2.
MA: microvascular area (μm^2) - average area of micro-vessels monitored in 5 microscopic fields magnified by 400x. obtained using the formula TMA/IMD.
MP: microvascular perimeter (µm) - average perimeter of micro-vessels obtained by monitoring 5 microscopic fields magnified by 400x. obtained using the formula TMP/IMD.
Ki-67 (%): average value obtained by the 3 evaluators.

Statistical analysis

Analyses were conducted in the Statistica software. The Spearman and Pearson tests were used to compare the positive rate of VM with tumoral grade, and to analyze the relationship between tumoral grade with quantified vascular parameters (IMD, TMA, TMP, average MA and MP) and Ki-67 proliferation marker. Significant level was set at 0.05.

Results

The subjects' age was between 2 and 13 years and tumor size varied from 0.35 to 5 cm in benign tumors and from 3 to 20 cm in malignant ones. Statistically significant correlations were established between tumor size and some angiogenic parameters such as micro-vessel count (IMD) ($r= 0.8$; $p< 0.001$). Malignant lesions were represented by different types of carcinomas

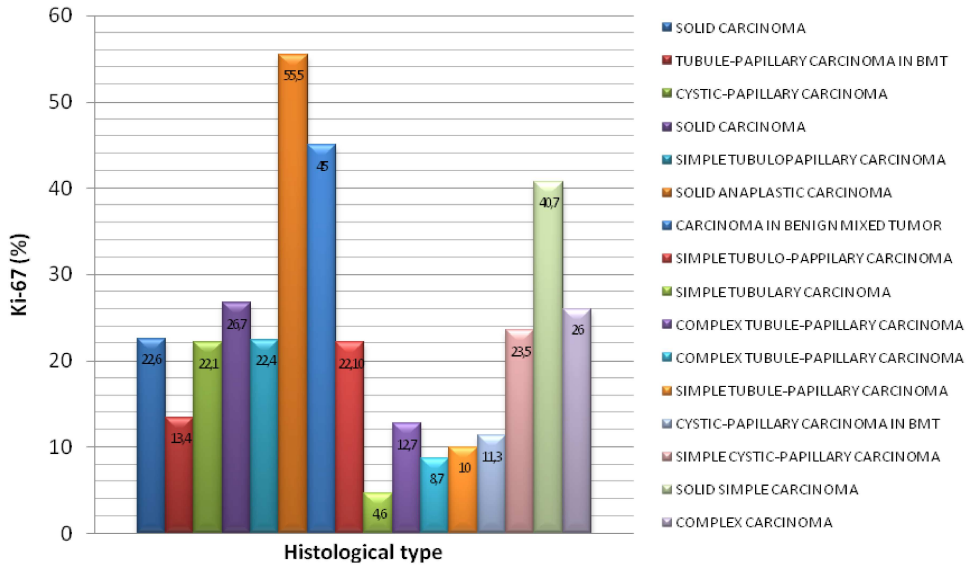

Figure 1. Ki-67 expression and histological type of canine mammary carcinomas

grouped into three histological grades (I - III). The standard characteristics of selected cases with mammary tumors related to classical prognostic factors are shown in *Table 1*.

Several critical differences regarding the distribution and expression of Ki-67 and CD31 biomarkers in benign and malign mammary tumors and their relation with classical prognostic factors have been suggested in our study. *Table 2* presents different aspects of Ki-67 and CD31 expression concerning intratumor neo-angiogenesis in canine mammary tumors, as defined by IHC.

For all studied mammary tumors excepting benign ones, Ki-67 nuclear biomarker revealed the proliferative potential of studied carcinomas (*Figure 3*). Nuclear staining was in general intense and moderate in neoplastic cells; immunomarked positive stromal nuclei were not counted. Concerning correlation between histological type and Ki-67 biomarker we had higher proliferation in solid canine mammary carcinomas (22.6-55.5% average Ki-67 values) confirming as well the poor prognosis for this histological type. The tumors with the lowest Ki-67 index were the following histological types: complex carcinomas, some simple carcinomas and carcinomas in benign mixed tumor (BMT) (4.69-13.4% average Ki-67 values), showing a reduced or moderate proliferative potential.

The other histological types, such as some complex and simple carcinomas and as well one carcinoma in BMT exhibit a great proliferative ratio, these tumors having 22.1 to 45% average Ki-67 values (*Figure 1*). According to the WHO classification, we noted significantly greater frequency of Ki-67 positivity in highly aggressive tumors such as grade II and III carcinomas. Thus, in grade II mammary tumors Ki-67 expression was positive in approximately 8.79% to 45.03% of tumor cells, respectively in 26.73-55.5% of tumor cells in poorly differentiated carcinomas (grade III). The lowest Ki-67 index was observed in grade I carcinomas (4.69% of tumor cells), but some others showed a high proliferation degree (22.13% of tumor cells). The data obtained confirm once again, if necessary, the utility of the Ki-67 nuclear antigen as a malignancy biomarker in canine mammary cancer. Furthermore, there was a direct statistical correlation between tumor grade and the proliferative potential of canine

Table 3. Correlation between tumor type and grade, average MA and MP, and Ki-67 biomarker

Tumor type	Average MA	Average MP	Average Ki-67 (%)
BT	496.98	102.72	-
	370.77	93.20	-
	167.21	66.25	-
	503.75	92.68	-
	536.19	111.38	-
G I	190.86	63.29	13.4
	248.20	84.13	22.13
	211.28	69.83	22.13
	110.72	52.83	4.69
G II	142.64	53.70	22.6
	137.4	51.55	22.43
	180.94	64.21	45.03
	167.28	63.46	12.72
	127.65	58.29	8.79
	177.25	63.33	10
	144.37	57.94	11.35
	98.54	46.59	23.59
	96.55	51.34	26
G III	96.66	42.98	26.73
	221.64	65.18	55.5
	88.02	46.17	40.71

BT - benign tumor;
G I - well-differentiated;
G II - moderately differentiated;
G III - undifferentiated.

mammary carcinomas (r=0.35; p<0.01).

CD31 expression was used to assess the angiogenic potential of the tumors studied. Our results show a correlation between IMD (intratumor microvessel density) and tumor grade, and between IMD and Ki-67 proliferation marker. Also, we had increased IMD in malign mammary lesions compared to benign tumors (4.6 to 6.6 IMD in benign tumors compared to 4.4 to 32.2 IMD in mammary carcinomas). Furthermore, a direct correlation between IMD and the histological grade in malignant lesions can be noticed. We obtained an increased IMD in grade II and III mammary carcinomas (10.14 to 32.2 IMD/microscopy field) comparatively with grade I mammary malignant lesions (4.4 to 12.71 IMD/microscopy field). We noted a progressive increase of intra-tumor micro-vessels expressed by IMD comparing to classical prognostic parameters, such as tumor type, grade and Ki-67 malignancy marker. Also, the number of newly formed micro-vessels in benign canine mammary tumors and differentiated G I carcinomas was reduced compared to moderately differentiated (G II) and undifferentiated (G III) carcinomas. In our study, IMD was correlated with malignancy established by tumoral grading (r=0.73; p<0.0001) and proliferation index Ki-67 (r=0.59; p<0.0005), showing as well its utility as malignancy marker.

We did not note a distinctive prognostic value of average TMA (total microvessel area (%)/microscopic image area) in selected areas (r= -0.04; p<0.9). Similarly with TMA, average TMP did not have statistic significance (r=0.28; p<0.46).

Concerning average MA and MP compared to tumor grade, our results show the following: a higher caliber of intra-tumor micro-vessels in G I carcinomas compared to G II and G III malignant tumors (showed by average MA and MP vascular parameters from *Table 3*). Also, we obtained a slightly smaller area (MA) and perimeter (MP) of micro-vessels along with increasing malignancy, showing as well high micro-vessel formation in G II and G III canine mammary carcinomas (88.0 until to 221.64 μm^2 average MA) comparing with G I ones (110.72 until to 248.2 μm^2 average MA). There is a significant correlation between average MA or MP and malignancy (r=0.55, p<0.0001; r= 0.63; p<0.0001).

Concerning vasculogenic mimicry (VM), we identified numerous blood channels without endothelium in vicinity of intratumoral necrotic areas (case 5, 17), as it is known that hypoxia is a stimulus for angiogenesis. Blood channels were located both within the sustain-

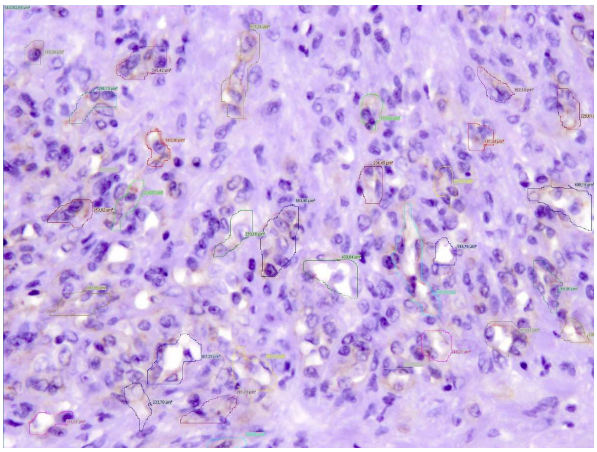

Figure 2. Solid carcinoma, grade II (case 1). Computerized semiautomatic evaluation of intratumoral neoangiogenesis; immunohistochemistry (IHC) anti-CD31, LSAB technique, Mayer's hematoxylin counterstaining, 350x.

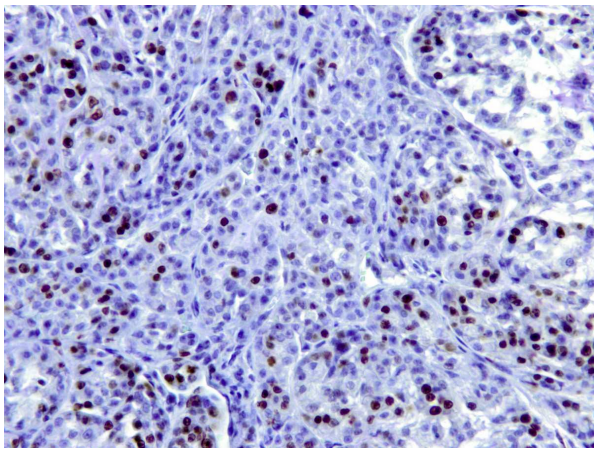

Figure 3. Carcinoma in benign mixed tumor, grade II (case 8); IHC anti-Ki-67 malignancy marker, LSAB technique, counterstaining with Mayer's hematoxylin, 200x.

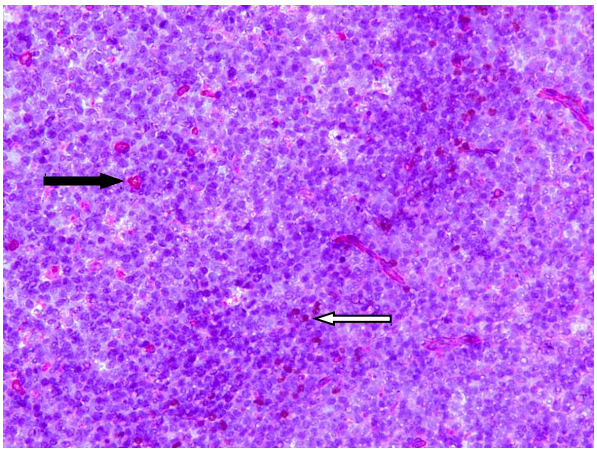

Figure 4. Solid mammary carcinoma, grade III (case 5). Blood channels (VM) with PAS positive material toward the lumen missing IHC reaction (black arrow); isolated immunomarked endothelial cells (blank arrow); double staining – IHC anti-CD31 and PAS reaction, counterstaining with Mayer's hematoxylin, 200x.

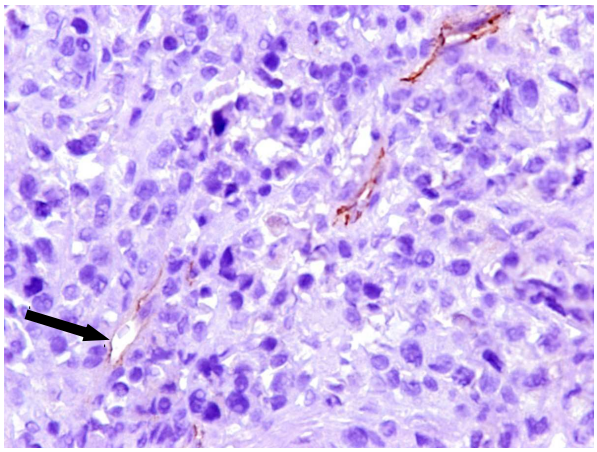

Figure 5. Anaplastic solid mamary carcinoma, grade III (case 7). Restricted IHC reaction to a limited portion of the vessel wall not to all vessel circumferences (arrow); IHC anti-CD31, LSAB technique, counterstaining with Mayer's hematoxylin, 400x.

ing stroma (cases 2, 4, 7, 18, 20) and the tumoral parenchyma (solid tumors with discreet stroma - cases 5, 7, 19) (*Table 2, Figure 4*). We noticed a VM-pattern in which the immunohistochemical reaction is discreet or more often restricted to a limited portion of the vessel wall instead of the whole circumference of the vessel (*Figures 5, 6*) (cases 4, 5, 6, 7).

The positive rate of VM was highest in G III canine mammary carcinomas compared to the other grades, and was practically absent in benign tumors. The VM pattern of intratumor neo-angio-

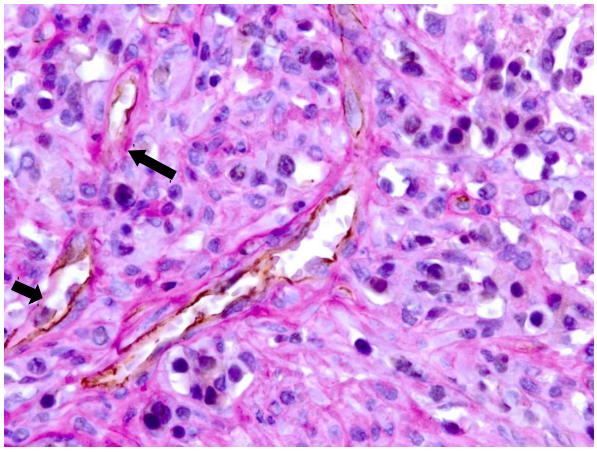

Figure 6. Anaplastic solid mamary carcinoma, grade III (case 7). Restricted IHC reaction to a limited portion of the vessel wall not to all vessel circumferences (arrows); double staining – IHC anti-CD31 and PAS reaction, counterstaining with Mayer's hematoxylin, 400x.

genesis became higher with the raise of histopathological grade: G I (frequently rare but present - +), G II (from total absence of VM pattern to frequently encountered - ++), and G III carcinomas (all cases ++). In addition, statistical analysis showed a significant correlation between tumor grade and VM positivity ($r=0.69$; $p<0.0001$). To elucidate the relationship between VM and Ki-67 nuclear proliferation marker, the VM-incidence was compared with the Ki-67 index. The VM-positive group had a higher incidence in carcinomas with a Ki-67 index greater than 8.7%. Nevertheless, there is one carcinoma with high Ki-67 index (45.03%) but without VM-positivity (*Table 4*). There was statistic correlation between the two parameters ($r=0.26$; $p<0.05$).

The highest incidence of VM was in solid carcinomas, all of them having VM-positivity (19.0% of cases), followed by simple carcinomas (19.0% of cases), carcinomas in BMT (9.5% of cases), and complex carcinomas (9.5% of cases). There were only two malignant canine mammary tumors that were not VM-positive, respectively one carcinoma in BMT and one simple carcinoma (9.5% of cases) (*Table 4*). In addition, the results show a direct correlation between IMD and VM. In this way, VM incidence was more frequent (++) in carcinomas with elevated IMD (10.4 to 32.2 vessels/microscopic field), rarely encountered but present (+) in malignant tumors with IMD ranging from 8.4 to 20.2 vessels/microscopic field, and absent (-) in malignant and benign tumors with decreased IMD (4.4 to 19.4 vessels/microscopic field). We obtained a statistic correlation between IMD and VM incidence ($r=0.47$; $p<0.0007$), showing as well an increased incidence of VM in canine mammary tumors with high rate of intratumoral neo-angiogenesis. Furthermore, there is a statistically significant correlation between VM and some other vascular parameters, such as average MA and MP ($r=0.39$, $p<0.003$; respectively $r=0.43$; $p<0.001$).

Discussion

Vasculogenesis is a complex multistage process characterized by the formation of new vessels from preexisting ones (1, 2, 5). Angiogenesis is essential for tumoral growing and metastasis, this is why in more aggressive tumors the angiogenesis is more intense due to increased demands for the newly formed structures. Excepting the main theories regarding intratumor angiogenesis, such as (I) The theory of multistage angiogenesis, and (II) the theory of cooption of preexisting vessels by the tumor, there is another one respectively, the theory of vasculogenic mimicry (1). The concept of vasculogenic mimicry has been introduced to define periodic acid-Schiff (PAS)-positive channels and loops lined by tumor cells, instead of endothelium, able to contribute to microcirculation. Previous studies have shown that the PAS-positive patterns are associated with a poor prognosis in several tumor types including uveal melanomas, osteosarcomas, etc. (6, 7, 8). This pattern of intratumor angiogenesis was noticed also in cell cultures originating from aggressive melanomas, where tumor cells have the ability to form PAS positive channels without endothelium (6). The generation of these patterns by highly invasive tumor cells suggests that PAS-posi-

Table 4. Incidence of vasculogenic mimicry pattern of intratumoral neo-angiogenesis

Tumor type	Histological diagnosis	Average Ki-67 (%)	IMD	Vasculogenic mimicry
Benign Tumors	Simple adenoma	-	6.6	-
	Fibroadenoma	-	5.0	-
	Simple adenoma	-	5.2	-
	Simple adenoma	-	5.5	-
	Simple adenoma	-	4.6	-
Malignant Grade I	Tubulopapillary carcinoma in BMT	13.4	8.4	+
	Simple cystic carcinoma	22.1	11.8	+
	Simple tubulopapillary carcinoma	22.1	12.7	++
	Simple tubulary carcinoma	4.6	4.4	-
Malignant Grade II	Simple solid carcinoma	22.6	20.2	+
	Simple tubulopapillary carcinoma	22.4	14	++
	Carcinoma in BMT	45.0	19.4	-
	Complex tubulopapillary carcinoma	12.7	10.4	++
	Complex tubulopapillary carcinoma	8.7	10.1	+
	Simple tubulopapillary carcinoma	10	11.1	+
	Cystic carcinoma in BMT	11.3	15.5	++
	Simple cystic carcinoma	23.5	15.6	++
	Complex carcinoma	26	11.8	+
Malignant Grade III	Simple solid carcinoma	26.7	15.8	++
	Solid anaplastic carcinoma	55.5	32.2	++
	Simple solid carcinoma	40.7	11.1	++

tive patterns are not remodeled blood vessels, as previously assumed (9, 10). Furthermore, by immunohistochemistry, looping PAS-positive patterns did not label with endothelial cell markers except focally, where they may anastomose with blood vessels (6, 11-14).

Although performed on a limited number of patients (21 female dogs with mammary tumors) and designed to evaluate intratumoral neo-angiogenesis (including IMD, TMA, MA, MP, and VM pattern) and its possible relation to classic prognostic factors (histological type, tumoral grade, Ki-67 nuclear proliferation biomarker), this study has pointed out several particular aspects of neo-angiogenesis in canine mammary tumors. While other studies bring into attention several biomarkers for canine mammary neoplasia including histological type and grade, mitotic index, products of tumoral suppressor genes (p53), apoptosis inhibitors (bcl-2), cells adhesion molecules (CD44), stem cell markers (CD34), and proliferation markers (AgNORs, Ki-67, PCNA) (1, 15-20), we used CD31 endothelial cell marker (for the quantification of neo-angiogenesis) and Ki-67 nuclear proliferation biomarker.

This study confirmed VM as a new type of blood supply in canine mammary carcinomas by using double staining. Angiogenesis (the formation or sprouting of endothelium-lined vessels from pre-existing vessels) and vasculogenesis (the differentiation of precursor cells into endothelial cells which develop de novo vascular networks) are two kinds of traditional blood types (21). Both have been reported in the canine mammary carcinomas examined. VM is a new pattern of matrix-rich networks surrounding tumor cells and was first reported in melanoma by Maniotis in 1999 (11). It refers to the de novo generation of

tumor microcirculation without participation by endothelial cells; it is independent of angiogenesis. The majority of research on VM focuses on mesenchymal tumor (22-24), while only a few concentrated on epithelial tumors (25-28). To date, there is dearth of research discussing canine mammary tumors. Thus, this study identifies intratumor neo-angiogenesis as a new prognosis factor and VM existence in canine mammary tumors. There are studies investigating the prognosis significance of IMD and VM pattern in breast cancers (29).

Our study evaluated intratumoral neo-angiogenesis in canine mammary cancer by using CD31 expression. We noticed a direct correlation between IMD and both Ki-67 proliferative biomarker and tumor type and grade. Research studies in literature reported statistically significant correlation between the nuclear proliferative marker Ki-67 and prognosis, showing its reliability as a quantitative prognostic factor (15, 16, 30). Also, micro-vessel density (or IMD) significantly increases from benign to malignant mammary lesions (15, 18). Furthermore, the study revealed an increased IMD in poorly differentiated mammary carcinomas (G II and G III) with high proliferative rate (high Ki-67 values), compared to differentiated ones (G I) that have low Ki-67 values. Our results are confirmed by statistic data that indicate a correlation between tumor grade and IMD ($r=0.73$; $p<0.0001$) and between proliferative index Ki-67 and IMD ($r=0.59$; $p<0.0005$). In addition, we studied some other possible markers of intratumoral neo-angiogenesis based on CD31 expression, such as average TMA, MA and MP. Also, comparing with bibliographic data that investigated only the prognostic significance of IMD (18), we tackle as well about some other parameters (TMA, MA and MP). Our findings did not show a distinct prognostic significance of TMA ($r= -0.04$; $p<0.9$) and TMP. On the other hand, MA and MP vascular parameters suggested the following: micro-vessel MA and MP significantly decreases along with increasing malignancy (mammary tumors with high Ki-67 values) and certainly vice versa. In the current study, we found that the undifferentiated canine mammary tumors have micro-vessels of small caliber as MA and MP suggested, and we noticed a statistic correlation between them and the tumor grade ($r=0.55$, $p<0.0001$; $r=0.63$; $p<0.0001$). Thus, intra-tumoral variation of the micro-vascular MA and MP has been shown to point out useful data regarding neo-angiogenesis especially in poor differentiated canine mammary carcinoma where the highest angiogenesis is reported.

There is still no affirmative conclusion on the prognostic significance of the endothelium markers among CD31, CD34 and CD105 (31). A long-term prognostic significance of angiogenesis in breast carcinomas compared to Tie-2/Tek, CD105, and CD31 immunocytochemical expression showed both CD31 and CD105 correlated with poorer survival (31). Mineo et al.'s study on lung cancer reported that CD34-IMD and tumor vessel invasion correlate with poor survival on multivariate analysis (32). Restucci et al. (2000) used CD31 marker to label intratumor microvascularization and concluded a direct correlation between IMD and canine mammary tumor malignancy. They highlighted an increased IMD in canine malignant tumors comparative with benign lesions (18). Similarly to our study, other studies have also reported IMD fidelity as a prognostic factor in different tumor types (2, 33-35).

On the other hand, there are some other studies indicating contradictions with presented results. As demonstrated by Luong et al. (36), IMD can't be used as prognostic factor. Moreover, the same authors have also suggested a more favorable prognosis in more vascularized tumors comparatively with poorly vascularized ones. Having these contradictions, further investigation needs to be performed in order to validate CD31 and IMD as prognostic biomarker in canine mammary cancer.

This study confirmed VM as a new type of blood supply in canine mammary tumors by double staining (CD31 endothelial marker and PAS reaction). By immunohistochemistry, looping PAS-positive patterns did not label with en-

dothelial cell markers except focally, where they may anastomose with blood vessels. We noticed a VM-pattern in which immunohistochemical reaction is discreet or more often restricted to a limited portion of the vessel wall, as demonstrated by other studies too (6, 12, 13). VM is an alternative type of blood supplement different from endothelium-lined vasculature. It is becoming evident that VM, the intratumor, tumor-cell-lined, patterned network, can provide an extra vascular fluid pathway, now known as the fluid-conducting meshwork (12).

VM in tumors plays an important role in tumor aggression as suggested by several studies (8, 37). In the current study, we identified that the positive rate of VM in canine mammary tumors was restricted as expected in carcinomatous lesions. VM existed in most malignant canine mammary tumors, with the highest frequency in G III and G II carcinomas, these grades having in fact increased Ki-67 values showing marked aggressivity. In accordance to the bibliography, the positive rate of VM increases with the increase of the histopathologic grade, which is consistent with a previous study of hepatocellular carcinoma (38). In addition, our results show a significant correlation between tumor grade of canine mammary tumors and VM incidence ($r=0.69$; $p<0.0001$), and between proliferative index Ki-67 and VM occurrence ($r=0.26$, $p<0.05$). Nasu et al's (39) in vitro study demonstrated that VM was linked to the aggressive tumor cell phenotype. From these studies emerge that the lower histopathologic grade with more cell heteromorphism can change cancer plasticity by genetic reversion to a pluripotent embryonic-like genotype to ultimately form VM (37).

We had some differences concerning VM incidence between histologic types, such as: highest incidence of VM in solid carcinomas, followed by simple carcinomas, carcinomas in BMT, and complex carcinomas. Previous research has demonstrated that VM existed in most tumors as a functional microcirculation correlated with poor clinical outcomes among tumor patients (38, 40). A retrospective study carried out on 203 patients with laryngeal squamous cell carcinoma showed that VM is associated with lymph node metastasis and histopathologic grade, the authors indicating an important role of VM in tumor progression (37). We have identified a relevant correlation between CD31 expression indicating IMD and VM. VM incidence was more frequent (++) in carcinomas with elevated IMD values, suggesting higher angiogenesis, higher potential of metastasis and poor prognosis ($r=0.47$; $p<0.0007$).

The VM-pattern of angiogenesis shows the plasticity and increased adaptability of tumoral cells to some injurious conditions such as hypoxia, contributing in this manner to intratumor blood flow. Those who rely on conventional markers of tumor "vascularity" as prognostic markers, and who are developing anti-cancer therapies by targeting angiogenesis should exercise caution concerning VM when interpreting their results. Vasculogenic mimicry is one example of the remarkable plasticity demonstrated by aggressive melanoma cells and suggests that these cells have acquired an embryonic-like phenotype (37). Larger cohort studies are necessary for the validation of CD31 as prognostic biomarker in canine mammary cancer. Further studies are needed to elucidate the specific molecular mechanism of VM in canine mammary cancer in order to explore new therapeutic targets, and to contribute to anti-vasculogenesis/angiogenesis therapy for vasculogenic mimicry in this type of cancer.

Conclusions

In conclusion, our results suggest that neo-angiogenesis (assessed by CD31 expression) and tumor proliferation rate (defined by Ki-67 nuclear marker) represent additional factors that promote tumor aggressiveness. We observed a significant correlation between the tumor grade and intratumor micro-vessel density (IMD) ($r=0.73$; $p<0.0001$), showing as well its

utility as malignancy marker in canine mammary carcinomas. Our results indicated a progressive increase of IMD in poorly differentiated mammary carcinomas with high proliferative rate (high Ki-67 values) ($r=0.59$, $p<0.0005$), compared to differentiated ones. While TMA parameter did not show a relation with malignancy, micro-vessel MA and MP significantly decrease along with increasing malignancy delivering useful data regarding intratumoral neoangiogenesis ($p<0.0001$).

Vasculogenic mimicry (VM) is an alternative type of blood supplement in carcinomatous canine mammary lesions. VM appeared more often in poorly differentiated canine mammary carcinomas, having a direct correlation with malignancy ($r=0.69$; $p<0.0001$). Occurrence of blood channels was higher in tumors with increased IMD ($r=0.47$; $p<0.0007$) suggesting higher angiogenesis, higher potential of metastasis and poor prognosis.

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Conflicts of interest. The authors declare that they have no conflicts of interest.

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