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Different role of COX-2 and angiogenesis in canine inflammatory and non-inflammatory mammary cancer

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ABSTRACT

Human inflammatory breast cancer (IBC) and canine inflammatory mammary cancer (IMC) are the most aggressive and fatal types of mammary cancer, and both have a very poor prognosis and low survival rate. Human IBC is characterised by exacerbated angiogenesis, lymphangiogenesis, and lymphangiotropism. Lymphangiotropism is also characteristic of IMC, but microvascular density (MVD) and lymphangiogenesis have not been previously studied in canine IMC. In this study immunohistochemical expression of several angiogenesis-related factors (cyclooxygenase [COX]-2, vascular endothelial growth factors A and D [VEGF-A, VEGF-D], and vascular endothelial growth factor receptor 3 [VEGFR-3]), MVD, lymphatic proliferation index (LPI), and Ki-67 tumour proliferation index (PI) were studied in 21 canine IMC samples, 20 canine high-grade malignant non-IMC mammary tumours (MMTs), and four normal mammary gland samples (NMGs).

All mammary neoplasms were histologically categorised as grade III. COX-2 values were also analysed by RT-PCR in seven IMCs, six MMTs and four NMGs. The expressions of COX-2, VEGF-A, and VEGF-D were significantly higher in IMC, MVD and LPI tumours, but not PI. In MMTs, COX-2 immunoexpression was significantly associated with VEGF-A, while in IMCs COX-2 was associated with VEGF-D (lymphangiogenic factor), its receptor VEGFR-3, and LPI. These results suggested that lymphangiogenic pathway stimulation isa specific role of COX-2 in IMC angiogenesis, which justifies the use of COX-2–based targeted palliative therapies in dogs. The exacerbated angiogenesis and lymphangiogenesis and the increased expression of angiogenesis-related factors further support canine IMC as a natural model for the study of human IBC.

Introduction

Inflammatory breast cancer (IBC) in women and inflammatory mammary cancer (IMC) in female dogs clinically resemble inflammatory processes such as dermatitis or mastitis (Tavassoli, 1999; Perez Alenza et al., 2001). They represent the most aggressive and fatal types of mammary cancer, with poor prognosis and survival rate in both species (Somlo et al., 1997; Victor et al., 1999; Clemente et al., 2010b). In spite of the macroscopic 'inflammatory' reaction, however, the infiltration of inflammatory cells is not a feature of IMC (Peña et al., 2003) or IBC (Tavassoli, 1999) at the microscopic level.

IBC and IMC present special molecular, clinical, and histopathological features (Jaiyesimi et al., 1992; Tavassoli, 1999; Perez Alenza et al., 2001; Giordano, 2003). Among the specific characteristics of IBC, exacerbated angiogenesis and lymphangiogenesis (Van der Auwera et al., 2004) and marked lymphangiotropism have been especially studied due to the importance of these features in metastatic spread. However, there are no reports regarding angiogenesis or lymphangiogenesis in IMC.

In spite of the histopathological type of tumour, IBC and IMC are characterised by high lymphangiotropism (Tavassoli, 1999; Peña et al., 2003; Van der Auwera et al., 2004). The massive invasion of dermal lymphatic vessels by neoplastic cells is the hallmark of

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the histopathological confirmation and causes the characteristic oedema by blocking lymph drainage (Tavassoli, 1999; Perez Alenza et al., 2001; Giordano, 2003; Peña et al., 2003). Moreover, vasculogenic mimicry phenomenon (formation of vascular channels lined by tumour cells) has been described in IBC (Kobayashi et al., 2002; Shirakawa et al., 2003) and IMC (Clemente et al., 2010a).

In canine mammary tumours, there is still little information regarding angiogenesis and the related angiogenic factors. Due to the reported general overexpression of vascular endothelial growth factor A (VEGF-A) in canine mammary cancer, the high levels of VEGF-A found in IMC (Millanta et al., 2010) should be confirmed by a proper comparison of IMC cases with poorly differentiated non-inflammatory canine malignant mammary tumours (grade III). There is no previously published information regarding vascular endothelial growth factor D (VEGF-D) and vascular endothelial growth factor receptor 3 (VEGFR-3) (lymphangiogenic factor and its receptor, respectively) in canine mammary neoplasms.

Cyclooxygenase (COX) enzymes catalyse prostaglandin formation from arachidonic acid. COX-1 is constitutively expressed in all tissues, whereas COX-2 participates in different steps of the carcinogenetic process and in tumour angiogenesis (Tsujii et al., 1998) and in the vasculogenic mimicry phenomenon in IBC (Basu et al., 2005, 2006). In canine mammary tumours, high expression of COX-2 is associated with malignancy (Dore et al., 2003; Mohammed et al., 2004; Queiroga et al., 2007), increased levels of VEGF-A (Queiroga et al., 2011), short survival (Lavalle et al., 2009) and angiogenesis (Queiroga et al., 2011). COX-2 was found to be increased in IMC tumour homogenates compared with malignant non-IMC mammary tumours (Queiroga et al., 2005).

In a recent study including 12 cases of canine IMC (de M Souza et al., 2009), high immunoexpression of COX-2 was reported, but findings were not compared with a corresponding non-IMC control group. Since no conclusive data are currently available on COX-2 expression, angiogenesis, and lymphangiogenesis in IMC tissue samples, the aims of this study were to describe COX-2 expression and its possible relation to angiogenesis and angiogenic growth factors (VEGF-A and VEGF-D) in IMC and canine high grade malignant non-IMC mammary tumours (MMTs) and to confirm whether angiogenesis and lymphangiogenesis are exacerbated in IMC, as in IBC.

Materials and methods

Animals and sampling procedure

Mammary tissue specimens were collected from 17 mixed-breed female dogs with malignant mammary tumours presented at the Veterinary Teaching Hospital) of the Complutense University of Madrid: 11 samples from spontaneous IMC (Trucut biopsies or necropsy samples) (IMC group) and six from spontaneous non-IMC, mammary tumours (surgical samples) (MMT group). Tru-cut biopsies of normal mammary glands ($n = 4$) from Beagle dogs were included as controls. In all specimens, two adjacent tissue fragments were separated and fixed in buffered formalin to be processed for histopathology and immunohistochemistry or preserved in RNA later (SIGMA) and frozen at −80 °C until use (for COX-2 mRNA expression analysis by quantitative PCR (qPCR)).

Twenty-four additional cases (10 IMC and 14 MMT) were selected from the paraffin archives and included in the study for COX-2, VEGFA, VEGFD, VEGFR-3, CD31, and Ki-67 immunohistochemistry. All animal procedures were approved by the institutional Ethics Committee.

Histopathology

For histopathology and immunohistochemistry studies, tissue samples (IMC neoplasms, $n = 21$; MMT neoplasms, $n = 20$) were fixed in formalin, routinely processed, and diagnosed following the World Health Organization classification system of canine mammary tumours (Misdorp et al., 1999). Clinical diagnosis of IMC was histologically confirmed by observing dermal lymphatic embolisation, thus only IMC cases with clinical and histological diagnoses were included (Perez Alenza et al., 2001). All tumours were histologically graded (Peña et al., 2012), and only grade III neoplasms were included in both groups. The presence of endothelial-like cells (vasculogenic mimicry) was assessed as previously described (Clemente et al., 2010a).

Immunohistochemistry

Immunohistochemistry of COX-2, VEGFA, VEGFD, VEGFR-3, CD31, and Ki-67 was performed on de-paraffinised sections, using a streptavidin–biotin-complex peroxidase method (Clemente et al., 2010a) after a high-temperature antigen unmasking protocol. In brief, slides are boiled in a pressure cooker for 2 min in buffer citrate, pH 6; the slides are subsequently cooled down at room temperature for 20 min, placed in distilled water and washed in Tris-buffered-saline (TBS).

Primary and secondary antibodies are detailed in Table 1. Canine mammary tumour slides with previously demonstrated reactivity to COX-2, VEGFA, VEGFD, VEGFR-3 antibodies were used as positive controls. Endothelial cells and mitoses were internal positive controls for CD31 and Ki-67 respectively. Corresponding negative controls were performed by substituting the primary antibody with a non-reacting antibody on canine tissues.

Evaluation of COX-2, VEGF-A, VEGF-D, and VEGFR-3 immunohistochemistry

Evaluation of the immunostaining was performed simultaneously by two independent observers. Due to the marked heterogeneity of the COX-2 immunostaining found in our series of cases, a new scoring method based on the percentage of immunostained cells in combination with the labelling intensity was developed. Thus, the tumours were scored in each case as follows: 0, negative, no staining; (1) low positive, weak diffuse cytoplasmic staining in 75–100% of the cells (may contain stronger intensity in less than 10% of the cancer cells); (2) moderate positive, moderate cytoplasmic staining in 50–100% of tumour cells, weak diffuse cytoplasmic staining in 75–100% of tumour cells with groups of cells (10–50%) with strong cytoplasmic staining, or only strong cytoplasmic staining in 10–50% of tumour cells; (3) intense positive, strong cytoplasmic staining in more than 50% of cancer cells.

VEGF-A, VEGF-D, and VEGFR-3 immunoreactions were scored in each case as negative (0) or positive when more than 10% of the tumour cells showed positive staining (Queiroga et al., 2011). Positive tumours were additionally categorised according to the intensity of immunostaining (1 = low positive, 2 = moderate positive, and 3 = intense positive).

Microvascular density, lymphangiogenic proliferation index, and tumour proliferation index

Microvascular density (MVD) and lymphangiogenic proliferation index (LPI) were simultaneously determined by two independent observers. MVD was assessed with sections immunostained with the endothelial marker CD31; each slide was first scanned at low power to identify the areas with the highest number of vessels ('hot spot' areas) (Weidner et al., 1991), generally located at the periphery of the neoplasm. Ten high-power fields with the highest number of blood vessels were counted. MVD was established as the mean value of the number of vessels (lymphatic and blood vessels, with less than 25 endothelial cells) counted on 10 high-power fields (40×). LPI was determined as the percentage of Ki-67 positive nuclei in 100 lymphatic endothelial cells, identified by morphology since the usefulness of lymphatic endothelial markers in canine mammary neoplasms is poorly documented and some markers do not show reaction (Sleeckx et al., 2012).

Lymphatic capillaries were distinguish from blood capillaries by a thin wall covered by a flatten endothelium, and the absence of red blood cells. Tumour proliferation index (PI), or proportion of Ki-67 positive neoplastic cells in each sample, was calculated using an image analyser (Peña et al., 1998).

RNA extraction and cDNA synthesis

Total RNA was extracted using a commercial kit (RNeasy Midi Kit, Qiagen) from mammary tissue samples. Once integrity was confirmed, the RNA was aliquoted and preserved in RNA secure Reagent (Ambion) at −80 °C. The isolated RNA was reverse-transcribed using iScriptTM cDNA Synthesis Kit (Bio-Rad) in a standard mixture containing 2 μL of total RNA in a 20 μL reaction volume.

Primer design

Primers were designed based on GenBank sequences for COX-2, glyceraldehyde-3-phosphate deshydrogenase (GAPDH), 18S rRNA, and β-actin using Primer3 software and the Oligo Analysis and Plotting Tool (QIAGEN). The COX-2 sequences were 5′-CTGTTCCCACCCATGTCAA-3′ and 5′-GCAGTTTTCGCCGTAGAATC-3′.

Analysis of mRNA expression by RT-PCR

Messenger RNA expression reactions were performed in an iCycler TM Thermal Cycler (Bio-Rad). Two microliters of each reverse-transcription reaction diluted 1/10 was amplified using DyNamo SYBR Green qPCR kit (Finnzymes). Buffer blanks contained all the reaction components without cDNA. All samples were run in duplicate. The cycling conditions were 95 °C for 8 min (stage 1) and 45 cycles at

Table 1
Immunohistochemistry of COX-2, VEGF-A, VEGF-D, VEGFR-3, and Ki-67 in canine normal and neoplastic malignant mammary gland: primary and secondary antibodies.[a]

Primary antibody			Dilution	Incubation	Secondary antibody		Dilution	Incubation
COX-2	N-20: sc-1746, Santa Cruz Biotechnology	Goat Pab	1:150	Overnight at 4 °C	Mouse anti-goat IgG biotinylated	Immunopure, 31732	1:200	30 min at RT
VEGF-A	A-20: sc-152, Santa Cruz Biotechnology	Rabbit Pab	1:500	Overnight at 4 °C	Swine anti-rabbit IgG biotinylated	E 0353, Dako	1:200	30 min at RT
VEGF-D	H-144: sc-13085, Santa Cruz Biotechnology	Rabbit Pab	1:500	Overnight at 4 °C	Goat anti-rabbit IgG biotinylated	E 0432, Dako	1:300	30 min at RT
VEGFR-3	FLT41-A, Alpha Diagnostic International	Rabbit Pab	1:200	Overnight at 4 °C	Goat multilink biotinylated antibody	MAD-001828QK, Master Diagnostica	Prediluted	10 min at RT
CD31	Clone: JC70A, Dako	Mouse Mab	1:20	Overnight at 4 °C	Goat multilink biotinylated antibody	MAD-001828QK, Master Diagnostica	Prediluted	10 min at RT
Ki-67	Clone: MIB; Master Diagnostica	Mouse Mab	1:50	1 h at RT	Horse anti-mouse IgG biotinylated	BA-2000, Vector	1:400	30 min at RT

[a] Pab, polyclonal antibody; Mab, monoclonal antibody; RT, room temperature.

95 °C for 30 s, 60.1 °C for 15 s, and 72 °C for 40 s (stage 2). In the final cycle (stage 3), the products were heated slowly to 95 °C to allow fluorescence measurements to be used to generate a dissociation curve.

The degree of product purity was assessed from the curve by confirming the presence of a single peak at the known product melting temperature and the absence of any primer dimers that might have generated a peak at a lower temperature (Ririe et al., 1997). Standard curves were produced from serial dilutions of samples to evaluate the PCR efficiency. The housekeeping genes used to seek variations due to RNA isolation in samples and PCR efficiency were 18S rRNA, GAPDH, and β-actin.

Statistical analysis

To compare the immunostaining of COX-2, VEGFA, and VEGFD and angiogenesis (MVD and LPI) in both groups (IMC vs. MMT) several variables were employed. COX-2, VEGFA, and VEGFD were categorical variables expressed as negative (0), low (1), moderate (2) and intense (3). These variables were also considered as negative (0) or positive (1, 2, or 3). MVD and LPI were numerical variables. Corresponding statistical analyses were performed using IBM SPSS 19 software. Data obtained by qPCR were standardized using qBase, which employs a delta-Ct relative quantification model with PCR efficiency correction and multiple reference gene normalisation (Vandesompele et al., 2002). For all statistical comparisons, values of $P < 0.05$ were considered significant.

Results

Histopathology

Different histological types of malignant mammary tumours were diagnosed in both groups (IMC and MMT) on stained sections (haematoxylin and eosin) as reported in a previous article using the same set of tumours (Clemente et al., 2010a). The 21 IMC neoplasms were classified as follows: simple tubular/papillary carcinoma ($n = 9$), lipid-rich carcinoma ($n = 5$), anaplastic carcinoma ($n = 4$), simple solid carcinoma ($n = 2$), and squamous cell carcinoma ($n = 1$).

Histological classification of the 20 non-inflammatory mammary cancer neoplasms (MMT) was as follows: simple/complex solid carcinoma ($n = 7$), carcinosarcoma ($n = 5$), anaplastic carcinoma ($n = 2$), malignant myoepithelioma ($n = 2$), simple tubular carcinoma ($n = 1$), complex tubulopapillary carcinoma ($n = 1$), sarcoma ($n = 1$) and osteosarcoma ($n = 1$). Of the 41 canine mammary cancers included, 7/21 IMC cases and 1/20 MMT cases showed highly malignant neoplastic cells resembling endothelial cells (ELCs, vasculogenic mimicry).

The histological diagnoses of the seven IMC included for COX-2 qPCR cases were: tubulopapillary carcinoma ($n = 3$), solid carcinoma ($n = 2$), anaplastic carcinoma ($n = 1$), lipid-rich carcinoma ($n = 1$). The histological diagnoses of the six non-inflammatory mammary cancer neoplasms (MMT) were as follows: solid carcinoma ($n = 2$), anaplastic carcinoma ($n = 1$), tubulopapillary carcinoma ($n = 1$), tubular carcinoma ($n = 1$), carcinosarcoma ($n = 1$).

COX-2 qPCR

COX-2 expression was highly increased in IMC cases (mean, 16.36; SD, 16.24) respect to MMTs (mean, 10.83; SD, 14.28) and NMGs (mean, 5.16; SD, 14.28). *P* values (0.004) denoted statistical differences between IMCs vs. MMTs vs. NMGs.

COX-2, VEGF-A, VEGF-D, and VEGFR-3 immunohistochemistry

Considering all IMC and MMT cases, 87.80%, 68.29%, 80.48%, and 65.85% of the samples expressed COX-2, VEGF-A, VEGF-D, and VEGFR3, respectively, by immunohistochemistry, while normal mammary gland samples and adjacent hyperplasias were all negative.

COX-2 immunolabelling was markedly heterogeneous. In both IMC and MMT, the cells that showed the highest cytoplasmic immunoexpression were: (a) highly malignant isolated neoplastic cells infiltrating the mammary gland stroma (Fig. 1); (b) neoplastic cells in emboli within the lumen of lymphatic vessels; and (c) endothelial-like tumour cells (ELCs) (highly aggressive tumour cells forming vascular channels similar morphologically to capillaries) (Fig. 1). COX-2 immunoexpression was higher in IMC cases vs. MMT cases ($P = 0.046$).

VEGF-A immunolabelling was cytoplasmic and homogeneous in neoplastic cells (Fig. 2), including ELCs. Few blood endothelial cells showed VEGF-A expression, and lymphatic endothelial cells were all negative. VEGF-A immunoexpression was higher in IMC cases vs. MMT cases considering the percentage of neoplasms with positive immunoreaction ($P = 0.011$) and the labelling intensity ($P = 0.006$). VEGF-D immunostaining was positive in the cytoplasm of neoplastic cells, with a homogeneous reaction in the same tu-

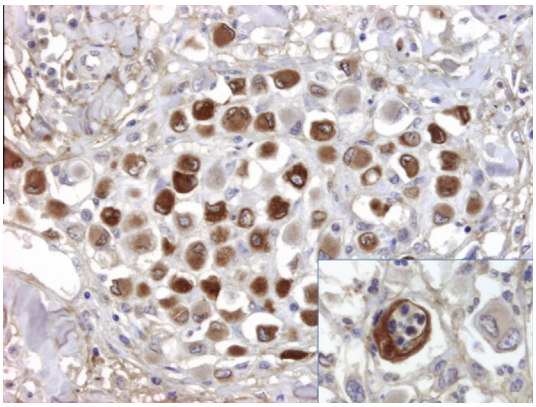

Fig. 1. Inflammatory mammary carcinoma with COX-2 immunohistochemistry showing COX-2–positive highly malignant infiltrating neoplastic cells and endothelial-like cells (ELC) expressing COX-2 (right bottom corner).

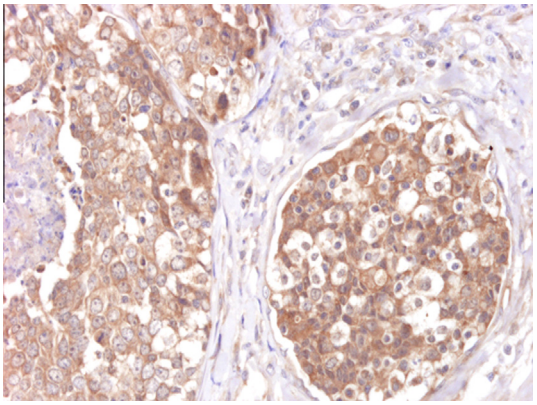

Fig. 2. Inflammatory mammary carcinoma with VEGF-A immunohistochemistry showing VEGF-A–positive neoplastic cells.

mour sample. In some IMC cases (7/21, 33.3%), VEGF-D cytoplasmic immunostaining was present as a focal, granular, and round distinct cytoplasmic formation. In the remaining IMC cases and in all MMT cases the VEGF-D cytoplasmic staining was diffuse (Fig. 3). All ELCs were positive. Blood and lymphatic endothelial cells were positive.

VEGF-D immunoexpression was higher in IMC cases vs. MMT cases considering the percentage of neoplasm with positive immunoreaction ($P = 0.001$) and the labelling intensity ($P < 0.001$). Neoplastic cells showed a slight to moderate cytoplasmic immunostaining for VEGFR-3 in IMC and MMT groups (Fig. 4). ELCs revealed the same intensity of immunoexpression as the rest of the neoplastic cells in the same tumour. Endothelial cells showed a variable positive (mostly lymphatic endothelial cells) and negative slight immunoreaction to VEGFR-3. There were no statistical differences between IMC and MMT groups for VEGFR-3 immunostaining.

Detailed information about COX-2, VEGF-A, VEGF-D, and VEGFR-3 immunoexpression is provided in Table 2.

MVD, LPI, and PI

MVD (Fig. 5) and LPI (Fig. 6) were significantly different in the three groups, with the highest values in IMC cases. Microvessels were frequently found in the marginal zone of the neoplasm (both IMC and MMT), showing a decreased number of vessels toward the core of the tumour. PI was not statistically different in IMCs with respect to MMTs. Detailed information regarding MVD, LPI, and PI is provided in Table 3.

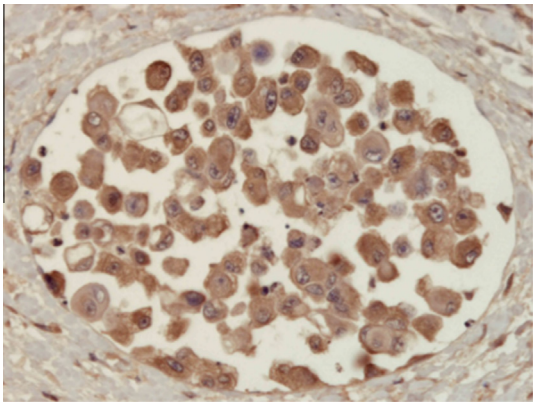

Fig. 3. Inflammatory mammary carcinoma with VEGF-D immunohistochemistry showing VEGF-D–positive neoplastic cells in lymphatic embolus.

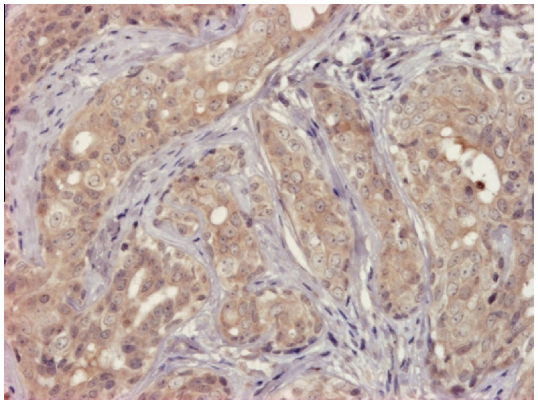

Fig. 4. Inflammatory mammary carcinoma with VEGFR-3 immunohistochemistry showing VEGFR-3–positive neoplastic cells.

Table 2
COX-2, VEGF-A, VEGF-D, and VEGFR-3 immunoexpression in normal mammary glands (NMGs), canine grade III malignant non-IMC mammary tumours (MMTs), and inflammatory mammary carcinomas (IMCs).

Intensity score[a]	NMGs		MMTs		IMCs		Total
	n	(%)	*n*	(%)	*n*	(%)	
COX-2 ($P = 0.04$)[b]							
0	4	(100.0)	3	(15.0)	2	(9.5)	9
1	0	(0.0)	9	(45.0)	6	(28.6)	15
2	0	(0.0)	7	(35.0)	4	(19.0)	11
3	0	(0.0)	1	(5.0)	9	(42.9)	10
VEGF-A ($P < 0.01$)[b]							
0	4	(100.0)	10	(50.0)	3	(14.3)	17
1	0	(0.0)	8	(40.0)	5	(23.8)	13
2	0	(0.0)	2	(10.0)	9	(42.9)	11
3	0	(0.0)	0	(0.0)	4	(19.0)	4
VEGF-D ($P < 0.01$)[b]							
0	4	(100.0)	8	(40.0)	0	(0.0)	12
1	0	(0.0)	11	(55.0)	6	(28.6)	17
2	0	(0.0)	1	(5.0)	12	(57.1)	13
3	0	(0.0)	0	(0.0)	3	(14.3)	3
VEGFR-3 ($P = 0.20$)							
0	4	(100.0)	9	(45.0)	5	(23.8)	18
1	0	(0.0)	9	(45.0)	10	(47.6)	19
2	0	(0.0)	2	(10.0)	6	(28.6)	8
3	0	(0.0)	0	(0.0)	0	(0.0)	0

[a] 0, negative; 1, low positive; 2, moderate positive; 3, intense positive.
[b] *P* values denote statistical differences IMCs vs. MMTs vs. NMGs.

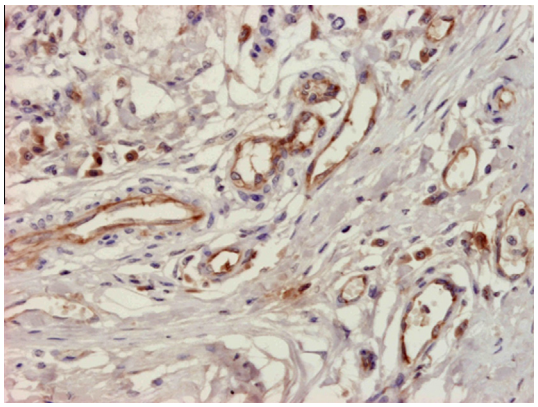

Fig. 5. Inflammatory mammary carcinoma with CD31 immunohistochemistry showing a 'hot spot' of angiogenesis with abundant CD-31 positive endothelial cells for counting microvascular density (MVD).

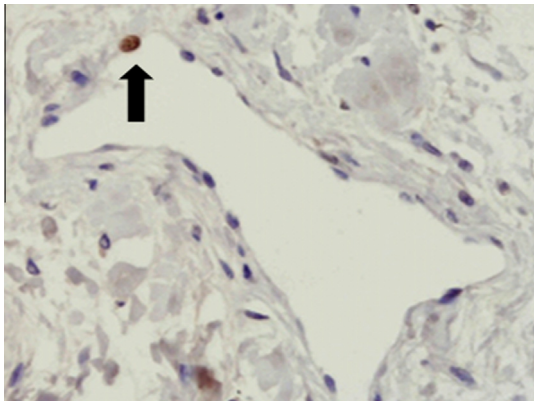

Fig. 6. Non-inflammatory mammary carcinoma with Ki-67 immunohistochemistry showing a lymphatic capillary with one positive nucleus in a lymphatic endothelial cell (arrow).

Association of COX-2 with angiogenesis in MMT and IMC groups

The associations of COX-2 with angiogenic factors were different in MMT and IMC groups as follows. In MMTs, COX-2 immunoexpression was significantly associated with VEGF-A ($P = 0.031$), while in IMCs COX-2 was associated with VEGF-D ($P = 0.001$), VEGFR-3 ($P = 0.017$), and LPI ($P = 0.013$). More information regarding significant statistical associations of angiogenic factors is provided in Table 4.

Discussion

The present study describes COX-2 expression in malignant high-grade canine mammary tumours and its association with angiogenesis. Our findings indicated that increased COX-2 expression is associated with the 'inflammatory' phenotype, showing a distinct role in IMC lymphangiogenesis. COX-2 has also been found to be overexpressed in IBC (Van der Auwera et al., 2004). In canine mammary tumours, high expression of COX-2 has been related with malignancy (Dore et al., 2003; Mohammed et al., 2004; Queiroga et al., 2007), microvessel density, and prognosis (Queiroga et al., 2005, 2010, 2011; Lavalle et al., 2009). To our knowledge, this is the first study in which COX-2 expression in IMC is described and is compared with high-grade canine MMTs. The PI measured by Ki-67 was not different in the two groups of high-grade tumours studied, indicating that both groups were also comparable in terms of proliferation.

COX-2 immunohistochemical expression and COX-2 mRNA expression were significantly higher in the IMC group than in the MMT group, as found in a previous study in which COX-2 was measured by enzyme immunoassay in tumour homogenates (Queiroga et al., 2005). The differing expression of COX-2 in IMC vs. non-IMC cases indicates a relevant role of COX-2 in the pathogenesis of IMC, probably via lymphangiogenesis. In both groups of our study, COX-2 was associated with MVD (which represents both blood and lymphatic vessels), although not significantly. According to a previous study (Queiroga et al., 2011), COX-2 over-expression correlated with VEGF-A in non-inflammatory canine mammary cancer. Our results regarding the association of COX-2 and VEGF-A ($P = 0.031$) to increased angiogenesis in MMTs agreed with previous reports in human breast cancer (Costa et al., 2002; Kirkpatrick et al., 2002).

In the present study, an association of COX-2 with VEGF-D (lymphangiogenic factor) and VEGFR-3 (VEGF-D receptor) was found in IMC cases, but not in MMT cases. Moreover, only the IMC group showed a significant association between VEGF-D and VEGFR-3 as well as an association between VEGFR-3 and lymphangiogenesis (LPI). In the IMC group, VEGF-A seemed to act mainly as a general growth factor increasing the PI ($P = 0.05$). According to our data, COX-2 could be responsible for the marked lymphangiogenesis of this specific type of mammary cancer through VEGF-D and VEGFR-3 because they are responsible for the growth of lymphatic endothelium (Achen et al., 1998; Skobe et al., 2001; Stacker et al., 2001). The association of COX-2 and VEGF-D and VEGFR-3 has not been reported in human IBC.

To our knowledge, this is the first report of VEGF-D immunohistochemical expression in canine mammary cancer and of angiogenesis and lymphangiogenesis in IMC. VEGF-D has not been studied in spontaneous human IBC either. Our results confirmed an exacerbated angiogenesis as a pathogenic mechanism of canine IMC as in human IBC (Van der Auwera et al., 2005). According to a previous study, IMC cases significantly over-expressed VEGF-A (Millanta et al., 2010) and VEGF-D, and presented higher MVD and lymphangiogenesis in comparison with high-grade malignant mammary neoplasms. The overexpression of VEGF-A in mouse IBC xenografts and VEGF-D in mouse IBC xenografts and human samples has been reported with different methodologies (Shirakawa et al., 2002a, 2003; Van der Auwera et al., 2004).

The exacerbated angiogenesis of IMC might explain the high frequency of vasculogenic mimicry in IBC (Shirakawa et al., 2002b) and IMC (Clemente et al., 2010a). A strong COX-2 positive immunoreaction was found in the cytoplasm of highly aggressive ELCs. COX-2 participation in the pathogenesis of vasculogenic mimicry has been previously demonstrated in human breast cancer (Basu et al., 2006). In our study, the presence of neoplastic cells forming vascular channels seemed to be related to high levels of angiogenic factors VEGF-A ($P = 0.011$) and VEGF-D ($P = 0.006$) as was suggested previously (Clemente et al., 2010a). VEGF signalling pathway has been demonstrated to trigger VM channels due to the high plasticity and lack of differentiation of highly aggressive tumour cells (Vartanian, 2012).

In spite of the important role of COX-2 in IMC, the use of selective COX-2 inhibitors as a palliative therapy in human IBC is hindered by cardiovascular side-effects (Robertson et al., 2010). However, the current study supports treating dogs with IMC with selective COX-2 inhibitors, which are drugs becoming increasingly popular in veterinary practice with low side effects.

Table 3
Microvascular density (MVD), lymphangiogenic proliferation index (LPI), and Ki-67 tumour proliferation index (PI) in normal mammary glands (NMGs), canine grade III malignant non-IMC mammary tumours (MMTs), and inflammatory mammary carcinomas (IMCs).

	NMGs			MMTs			IMCs			*P*
	n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	
MVD	4	4.9	0.59	20	11.84	4.10	21	16.04	5.38	0.01[a]
LPI	4	0.23	0.47	20	8.34	7.11	21	15.21	9.68	0.02[a]
PI	4	2.61	1.52	20	30.18	12.83	21	37.64	11.13	0.08

[a] *P* values denotes statistical differences IMCs vs. MMTs vs. NMGs.

Table 4
P values for statistically significant associations of COX-2, tumour proliferation index (PI), lymphatic proliferation index (LPI), and angiogenesis-related factors incanine grade III malignant non-IMC mammary tumours (MMTs) and inflammatory mammary carcinomas (IMCs).

	COX-2	VEGF-A	VEGF-D	VEGFR-3	PI	LPI
COX-2		0.031[a]	0.001[b]	0.017[b]		0.013[b]
VEGF-A	0.031[a]				0.05[b]	
VEGF-D	0.001[b]			0.001[b]		
VEGFR-3	0.017[b]		0.001[b]			0.005[b]
PI		0.05[b]				
LPI	0.013[b]			0.005[b]		

[a] Significant association in MMTs.
[b] Significant association in IMCs.

Conclusions

A differential profile of VEGF-A, VEGF-D, and COX-2 between IMC and MMT canine mammary tumours exists, suggesting that COX-2 mediates a different mechanism in angiogenesis and lymphangiogenesis pathways in IMC. The increased angiogenesis, lymphangiogenesis, and angiogenic factors (VEGF-A and VEGF-D) observed in IMC cases additionally support the canine IMC as a natural model for the study of human IBC.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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