Prognostic value of vascular endothelial growth factor and hypoxia-inducible factor 1α in canine malignant mammary tumors

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Abstract. Mammary tumors are the most common type of tumor in dogs, with approximately half of these tumors being malignant. Hypoxia, characterized by oxygen levels below normal, is a known adverse factor to cancer treatment. The hypoxia-inducible factor 1α (HIF-1α) is a central regulator of the pathophysiological response of mammalian cells to low oxygen levels. HIF-1α activates the transcription of vascular endothelial growth factor (VEGF), which in turn promotes angiogenesis through its ability to stimulate the growth, migration and invasion of endothelial cells to form new blood vessels, contributing to tumor progression. In this study, we evaluated the serum concentration and gene expression of VEGF and HIF-1α linking them with clinicopathological parameters and survival of dogs with mammary tumors in order to infer the possible prognostic value of these factors. We collected blood and tumor fragments of 24 female dogs with malignant mammary tumors (study group) and 26 non-affected female dogs (control group) to verify the gene expression of VEGF and HIF-1α by quantitative real-time PCR (qPCR) and the serum levels by ELISA (enzyme-linked immunosorbent). The results showed high serum levels of VEGF in the study group and its correlation between abundant vascularization, lymph node involvement, metastasis, death rate and low survival ($p<0.05$). The serum percentage of HIF-1α in female dogs with mammary neoplasia was lower than that in the control group and higher in female dogs with tumor metastasis and history of tumor recurrence ($p<0.05$). Regarding gene expression, there was a gene overexpression of VEGFA in female dogs with poor outcome, in contrast to the gene underexpression of HIF-1A. Taken together, these results suggested that VEGF is important in tumor progression and can be used as a potential prognostic marker in the clinic and may be useful in predicting tumor progression in dogs with mammary neoplasia.

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Introduction

Mammary tumors are the most common neoplastic process in female dogs, representing ~52% of neoplasms, of which 41-53% are histologically diagnosed as malignant, with distant metastasis being the most common cause of death in female dogs (1-6). Canine mammary tumors (CMTs) are similar to human breast cancers (5,7), thus they are a suitable animal model for the study of mammary carcinogenesis in the two species (2,8).

Prognosis is directly associated with factors such as tumor size, lymph-node involvement, presence of distant metastasis, histological type, histologic grade and intravascular growth (9). Therapy based on prognostic assessment enables the application of different therapeutic modalities used in cancer treatment with the intensity and effectiveness appropriate for each patient, increasing survival (10,11).

Angiogenesis is a process in which new blood vessels are formed from pre-existing vasculature (12), which is necessary

to supply nutrients and maintain homeostasis in the tissues of the body (13). This process has been shown to be a necessary process for oncogenesis as well, in addition to subsequent tumor growth and dissemination through metastases (14).

Angiogenesis is regulated by a number of pro- and anti-angiogenic factors, such as vascular endothelial growth factor (VEGF) and hypoxia-inducible factor (HIF) (15,16). These factors, which are released by tumor cells promote activation, proliferation and migration of endothelial cells to tumor tissue, allowing for the rapid formation of functional neovasculature (17).

HIF transcription factors mediate the primary transcriptional response to hypoxic conditions in normal and neoplastic cells. HIFs form heterodimeric complexes composed of the α and a stable β subunit. Together these subunits bind hypoxia response elements (HREs) to hundreds of genes that facilitate the adaptation to hypoxia, specifically promoter elements present in the promoter region of VEGF (18,19).

HIF-1α is an oxygen-liable subunit, that in normal oxygen conditions is maintained at a low level (20,21) as it is recognized by the Hippel-Lindau tumor suppressor (pVHL) and degraded by the proteasome (22-24). Under hypoxic conditions, pVHL binds to nitric oxide (NO) and the HIF-1α is not recognized, allowing the migration of HIF-1α from the cytoplasm to the nucleus, therebey inducing the expression of VEGF (15,16,25-28).

VEGF and its receptors are confirmed signaling pathways in angiogenesis (29). VEGF stimulates endothelial cell proliferation, migration and capillary tube formation (28,30-31). Tumor growth through angiogenesis is directly correlated with VEGF expression in breast cancer (15,16,32-34). It was reported that VEGF expression in canine and feline mammary carcinomas is associated with a more aggressive behavior and a poor diagnosis/prognosis (35-37).

The aims of this study were to determine the serum levels and gene expression of VEGF and HIF-1α in female dogs with malignant mammary neoplasia, and to verify their correlation with clinicopathological parameters and clinical evolution, aiming to determine its prognostic value.

Materials and methods

Ethical considerations. This study was approved by the Ethics Committee of Sao Jose do Rio Preto Medical School (protocol no. 5230/2010).

Sample characterization. Peripheral blood samples and tumor fragments from 24 female dogs with malignant mammary neoplasia (study group) and 26 female non-affected dogs (control group) were collected at the veterinary clinics in São José do Rio Preto, SP, Brazil and the surrounding region between 2011 and 2012. Exclusion criteria for the control group were rigorously followed and included females with no tumor history and no detectable disease inflammation/infections in the period prior to the sampling.

Female dogs from the study group were evaluated by the veterinary with respect to physical (age and breed), pathological (time-course, the interval between tumor diagnosis and surgical removal, tumor localization, lymph-node involvement, tumor mass size, clinical staging, ulceration and vascularization) and clinical (metastasis, local recurrence and death) characteristics.

Following the tumor excision and blood collection, the animals of study group were followed for 12 months. During this period the presence of local tumor recurrence, metastasis (confirmed by X-ray) and death were described by the veterinary, allowing the determination of survival time and disease-free survival time (the time from excision until the detection of metastasis and/or recurrence).

The tumor fragments collected at excision were divided into two sections: The first one was fixed in buffered 10% formaldehyde for 24 h and paraffin-embedded. Histological sections (3 μm) were obtained and stained with hematoxylin and eosin (H&E) by standard histological procedures for histopathology. The second section was immersed in RNA stabilizing solution, RNAlater (Invitrogen Life Technologies, Eugene, OR, USA) for qPCR analysis.

The parameters employed for the histological classification were performed according to the Canine Mammary Neoplasms Histological Classification, modified from Misdorp *et al* (38,39) and the clinical staging system (TNM) of canine mammary carcinomas established by Owen (40).

Most of the malignant tumors consisted of simple carcinoma, such as tubulopapillary carcinoma type (16/24) (67%). The age range of the animals was 7-14 years (mean, 10 years) and 25% of animals were of indeterminate breed. Among the clinicopathological characteristics, there was a predominance of time course >6 months (11/24) (46%), tumors with multiple location (14/24) (58%), lymph-node involvement N0 (17/24) (71%), tumor mass size <3 cm (12/24) (50%), clinical staging I (10/24) (42%), tumors without ulceration (20/24) (83%) and moderate vascularization (16/24) (67%). The local recurrence rate was 17%, metastasis 25% and death 33%. The patient characteristics are shown in Table I.

Enzyme-linked immunosorbent assay (ELISA). For the enzyme-linked immunosorbent assay, the blood (3 ml) was collected in a CORVAC serum separator tube (Labor import, São Paulo, SP, Brazil) containing clot activation additive and barrier gel, stored at 4˚C, processed by centrifugation (1,000 x g, 25 min) and passed through a 13-mm serum filter to remove potentially contaminating cells. The serum was immediately cryopreserved at -80˚C (7).

Quantification of serum VEGF. VEGF content was determined by using a Quantikine Canine VEGF Immunoassay kit (R&D Systems, Minneapolis, MN, USA). A specific monoclonal antibody anti-canine VEGF was pre-coated onto a 96-well polystyrene microplate, and 100 μl of buffered protein solution, standards and samples were pipetted into the wells. After incubation for 2 h, the microplate was washed three times with 300 μl/well. The reaction also included incubations at room temperature with 200 μl/well of enzyme-linked polyclonal antibody against VEGF conjugated to horseradish peroxidase for 2 h followed by another wash and 200 μl/well of substrate solution (H_2O_2 and tetramethylbenzidine) for 25 min. Then, 50 μl of stop solution (2 N sulfuric acid) was added to each well and the optical density (OD) was measured at 450 nm in a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The reaction intensity was proportional to the

Table I. Mean serum concentration of VEGF and serum percentage of HIF-1α and its correlation with clinicopathological parameters.

Clinicopathological parameters	Number of dogs	VEGF (pg/ml)	HIF-1α (% of control)
Clinical feature			
Control	24	51.77±4.875	100.0±5.442
Samples	26	136.4±48.79	78.51±3.146
P-value		0.03[a]	0.001[a]
Age			
<10 anos	11 (46%)	79.95±35.46	84.83±5.193
≥10 anos	13 (54%)	161.3±78.59	73.16±3.290
p-value		0.37	0.06
Tumor location			
Multiple	14 (58%)	146.2±71.87	77.89±4.850
Single	10 (42%)	96.63±52.86	79.38±3.606
P-value		0.59	0.82
Time course			
1 month	4 (17%)	22.27±12.51	75.93±4.763
Up to 6 months	9 (37%)	144.5±64.98	78.53±3.796
More than 6 months	11 (46%)	138.7±84.96	79.43±6.119
P-value[b]		p>0.05	p>0.05
Tumor mass size			
T1	12 (50%)	155.3±79.83	78.63±3.607
T2	4 (17%)	132.8±108.6	89.10±14.11
T3	8 (33%)	63.40±6.664	71.63±2.902
P-value[b]		p>0.05	p>0.05
Lymph node involvement			
N0	17 (71%)	33.13±6.188	77.30±2.646
N1/N2	7 (29%)	176.7±68.16	80.93±8.144
P-value		0.01[a]	0.59
Metastasis			
M0	18 (75%)	45.64±9.238	75.79±2.545
M1/M2	6 (25%)	201.6±96.53	94.47±12.73
P-value		0.01[a]	0.02[a]
Clinical staging			
I/II	12 (50%)	85.51±59.44	78.79±3.487
III/IV	12 (50%)	104.7±45.72	77.30±5.352
P-value		0.80	0.81
Ulceration			
Yes	4 (17%)	225.6±168.9	73.35±4.272
No	20 (83%)	95.32±36.62	79.54±3.670
P-value		0.24	0.47
Vascularization			
Abundant	8 (33%)	247.7±121.4	77.38±7.972
Moderate	16 (67%)	41.35±9.111	79.07±2.807
P-value		0.02[a]	0.80
Recurrence			
Yes	4 (17%)	41.70±8.763	98.88±16.26
No	20 (83%)	137.9±52.20	75.90±2.524
P-value		0.44	0.01[a]
Censorship			
Death	8 (33%)	238.1±92.95	79.72±8.220
Alive	16 (67%)	38.81±9.467	77.90±2.633
P-value		0.02[a]	0.79

[a]Significant p-value; [b]ANOVA.

concentration of VEGF. The calculation of OD was determined through the adjustment curve four parameter logistic (4-PL), using the software SkanIt for Multiskan FC 2.5.1 (Thermo Fisher Scientific).

Quantification of serum HIF-1α. HIF-1α content was determined by using a HIF-1α Transcription Factor Assay kit (Abnova, Taipei, Taiwan). First, 90 µl/well of complete transcription factor binding assay buffer (CTFB) (composited by UltraPure Water, 4X transcription factor binding assay buffer concentrate, transcription factor reagent A and 300 mM dithiothreitol, DTT), and 10 µl of transcription factor HIF-1α-positive control were mixed with samples in the appropriate wells. After overnight incubation at 4˚C, the microplate was washed five times with 200 µl/well. The antibodies employed were transcription factor HIF-1α primary antibody 1:100 in 1X antibody binding buffer (ABB) and transcription factor goat anti-rabbit HRP-conjugated secondary antibody 1:100 in 1X ABB. The reaction also included subsequent incubations at room temperature with 100 µl/well of transcription factor developing solution (chromogenic substratum) for 15-45 min. OD was measured at 450 nm in a microplate reader (Thermo Fisher Scientific). The reaction intensity was proportional to the concentration of HIF-1α. The mean HIF-1α serum absorbance of the control group was established as 100%, the serum percentage of HIF-1α in the study group being calculated in relation to the control group.

Quantitative PCR (qPCR)

Sample processing. Tumor samples were collected in a falcon tube of 15 ml containing RNA-stabilizing solution, RNAlater (Invitrogen Life Technologies), stored at room temperature for 24 h, manually processed using a razor into 100 mg/section, immersed in TRIzol reagent (Invitrogen Life Technologies) and macerated for total RNA extraction, according to the manufacturer's instructions. The RNA concentration of each sample was determined with a NanoDrop 2000 (Thermo Fisher Scientific), and RNA integrity was confirmed on a 1% agarose gel. The RNA from each sample was reverse-transcribed to complementary DNA (cDNA) using a High Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA).

Gene expression of VEGF and HIF-1α. The standard curve was calculated, and analyses for the differential expression of HIF1A or VEGFA and endogenous control genes *RPS19* and *RPL8* were performed in triplicate using StepOnePlus System (Applied Biosystems) and TaqMan Universal Master mix (Applied Biosystems), as recommended by the manufacturer. Each transcript level was normalized by division with the expression values of *RPS19* and *RPL8* used as an endogenous control. The assays used were HIF-1α (Cf02741632_m1), VEGFA (Cf02623449_m1) and RPL8 (Cf02663820_m1) and the RPS19 primer sequences used for amplification were: sense (5'-GCC TTC CTC AAA AAG TCT GGG -3'), antisense (5'-GCT TGC TCC CTA CGA TGA GAA C-3') and probe (5'-CCC TGA ATG GGT GGA C-3') (Applied Biosystems).

Each reaction consisted of 10 µl of Master Mix, 1 µl of TaqMan, 8 µl of DEPC water and 1 µl of cDNA (100 ng/ml). The amplification scheme appointed was: 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The expression of each gene of interest was calculated by the quantification method related to the average of the normalizing genes used as endogenous controls (ΔΔCt) (41).

Statistical analysis. Statistical analysis was performed using GraphPad Prism4 (San Diego, CA, USA) and Stats Direct (London, UK) software. The results were previously submitted to descriptive analyses for the determination of normality and were considered to have a normal distribution. The different clinicopathological characteristics were separated in groups and compared by the Student's t-test or ANOVA, followed by the Bonferroni test. The values were presented as mean ± standard deviation (SD).

The survival curve was constructed following the Kaplan-Meyer method. The cut-off points for the VEGF and HIF-1α levels were established by the receiver operating characteristic (ROC) curve. For the ROC curve, the percentage of VEGF and HIF-1α of female dogs who died was compared with that of dogs that survived until the end of the follow-up period. Survival curves were plotted by the Kaplan-Meier method and the differences between the curves were assessed using a log-rank test and hazard function.

Analysis of tumor vascularization by double staining immunohistochemistry. Tumor vascularization was analyzed by the veterinarian at the moment of surgery, based on macroscopic visualization of the vessels involved in the tumor and it was subsequently confirmed by the pathologist during the histopathological examination. According to this observation, the samples were classified as moderate and abundant vascularization. To confirm this analysis, we performed a double staining immunohistochemical technique from the analysis of the presence of CD34, a transmembrane glycoprotein whose expression is associated with hematopoietic precursors and capillary endothelial cells, and intermediate-purity plasma-derived Factor VIII. Images were captured using Nikon eclipse E200 (Nikon Instrument Group, Melville, NY, USA).

Tissue microarray technique (TMA). The double-labeling immunohistochemical technique was performed according to Maschio *et al* (42). The tumor fragments were fixed in formalin and embedded in paraffin and assembled as a tissue microarray (TMA). TMA consisted of representative areas of the tumors removed from the donor block and added to the receiver block in duplicate representing different regions of the tumor, via the manual tissue arrayer 1 (Beecher Instruments Micro-array Technology, Silver Spring, MD, USA). Sections (3 µm) from blocks of TMAs were deposited on electrostatically charged slides (StarFrost Waldemar Knittel GmbH, Brunswick, Germany). A map containing the location of each fragment in TMAs was constructed using Microsoft Excel (Microsoft Co., Redmond, WA, USA).

The double-labeled reactions were performed in automated immunostaining equipment (Ventana Bench Mark XT, Roche Diagnostics, Mannheim, Germany), using anti-CD34 and anti-Factor VIII antibodies (Table II). The display systems used in the reactions of double-labeled Factor-VIII and CD34 were Enhanced Alkaline Phosphatase Red Detection and iVIEW DAB Detection, respectively.

Table II. Information about antibodies used for double staining immunohistochemistry.

Antibody	Specificity	Clone	Dilution	Company	Positive controls
CD34	Monoclonal (mouse)	QBEnd 10	0:500	Dako	Breast normal tissue
Factor VIII	Polyclonal (rabbbit)	-	0:200	Dako	Tonsil tissue

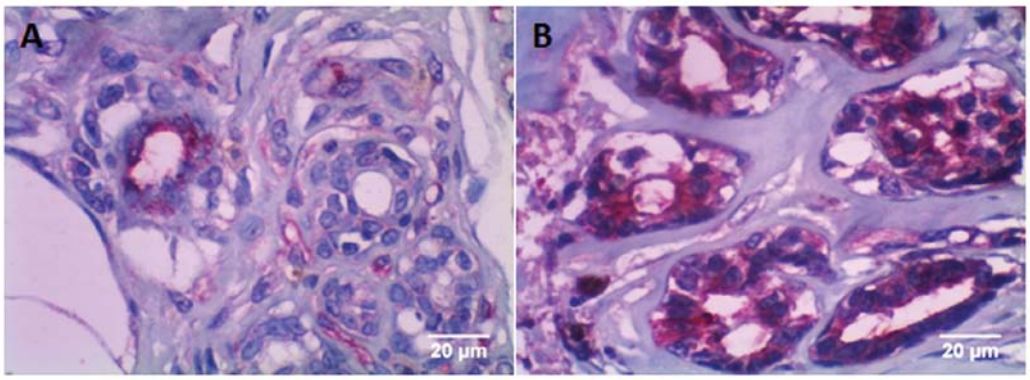

Figure 1. Immunohistochemical staining in malignant mammary tumors of dogs using double-staining immunohistochemistry for anti-CD34 (membrane in brown color) and anti-Factor VIII (cytoplasm in red color), present in tumor blood vessels. Magnification, x40. (A) Moderate and (B) abundant tumor vascularization.

Deparaffinization was initiated by applying EZPrep reagent (Roche Diagnostics) and heating at 75˚C for 8 min. For antigen retrieval, cell conditioner (Roche Diagnostics) was applied for 8 min at 95˚C and then 64 min at 100˚C. The slides were washed with reaction buffer (Roche Diagnostics) and incubated for 4 min in this solution. Then, UV inhibitor (Roche Diagnostics) was applied for 4 min and the slides were washed with reaction buffer. The anti-CD34 primary antibody was incubated for 1 h and the slides were washed with reaction buffer. UV UNIV MULT HRP (Roche Diagnostics), UV DAB (Roche Diagnostics) and H_2O_2 UV DAB reagents (Roche Diagnostics) were then applied for 8 min each, respectively. Copper UV reagent (Roche Diagnostics) was applied and the slides were heated to 90˚C for 4 min and 37˚C for an additional 4 min. Then, 100 ml of the primary antibody anti-Fator VIII was applied and incubated for 1 h. The UV Red UNIV MULT (Roche Diagnostics) was incubated for 12 min. Subsequently, UV Red Enhancer (Roche Diagnostics) was applied for 4 min. Reagents UV Fast Red A (Roche Diagnostics), Naphthol Red UV (Roche Diagnostics) and UV Fast Red B (Roche Diagnostics) were applied for 8 min each. After this procedure, the slides were washed with reaction buffer and incubated with Hematoxylin II reagent (Roche Diagnostics) for 8 min. The slides were washed with reaction buffer and incubated with bluing reagent (Roche Diagnostics) for 4 min and again washed with reaction buffer.

The staining of Factor-VIII antibody was observed in the cytoplasm as red, and CD34 antibody in the cytoplasmic membrane as brown (Fig. 1).

Results

Correlation between clinicopathological parameters and serum levels. The mean serum concentration of VEGF at the surgical excision moment in female dogs with mammary tumors was significantly higher than in the control group (136.4 pg/ml vs. 51.77 pg/ml; p=0.03; Table I). By contrast, the serum percentage of HIF-1α was significantly higher in the control group (100.0 vs. 78.51%; p=0.001; Table I).

The univariate analysis showed that the VEGF serum concentration was significantly higher in dogs with highly vascularized tumors (p=0.02; Table I), female dogs with lymph node involvement (p=0.01; Table I), metastasis (p=0.01; Table I) and in those that died within the follow-up period (p=0.02; Table I). Furthermore, the HIF-1α levels were significantly higher in female dogs with metastasis (p=0.02; Table I) and tumor recurrence history (p=0.01; Table I).

As far as the survival curve is concerned, the samples were divided according to the last VEGF and HIF-1α serum values measured prior to death, with a cut-off value of 75,483 pg/ml [sensitivity (95% CI) = 75%, specificity (95% CI) = 94%] for VEGF and a cut-off value of 87,179 percentage of control [sensitivity (95% CI) = 38%, specificity (95% CI) = 81%] for HIF-1α. This procedure demonstrated a negative correlation between VEGF concentration and survival time (OR, 9.169; CI 95% = 5.266-201.8; p=0.0002). No correlation was observed between HIF-1α serum percentage and survival time (OR, 1.471; CI 95% = 0.3417-7.320; p=0.34) (data not shown).

Correlation between clinicopathological parameters and gene expression. To examine the effect of gene expression of VEGF and HIF-1α to the spread of the disease we analyzed the correlation between the gene expression and clinical evolution. The qPCR analysis revealed that VEGF was significantly overexpressed in female dogs with highly vascularized tumors (p=0.03; Fig. 2A), lymph-node involvement (p=0.02; Fig. 2B), metastasis (p=0.04; Fig. 2C), tumor recurrence (p=0.0002; Fig. 2D) and death (p=0.04; Fig. 2E). The HIF-1α

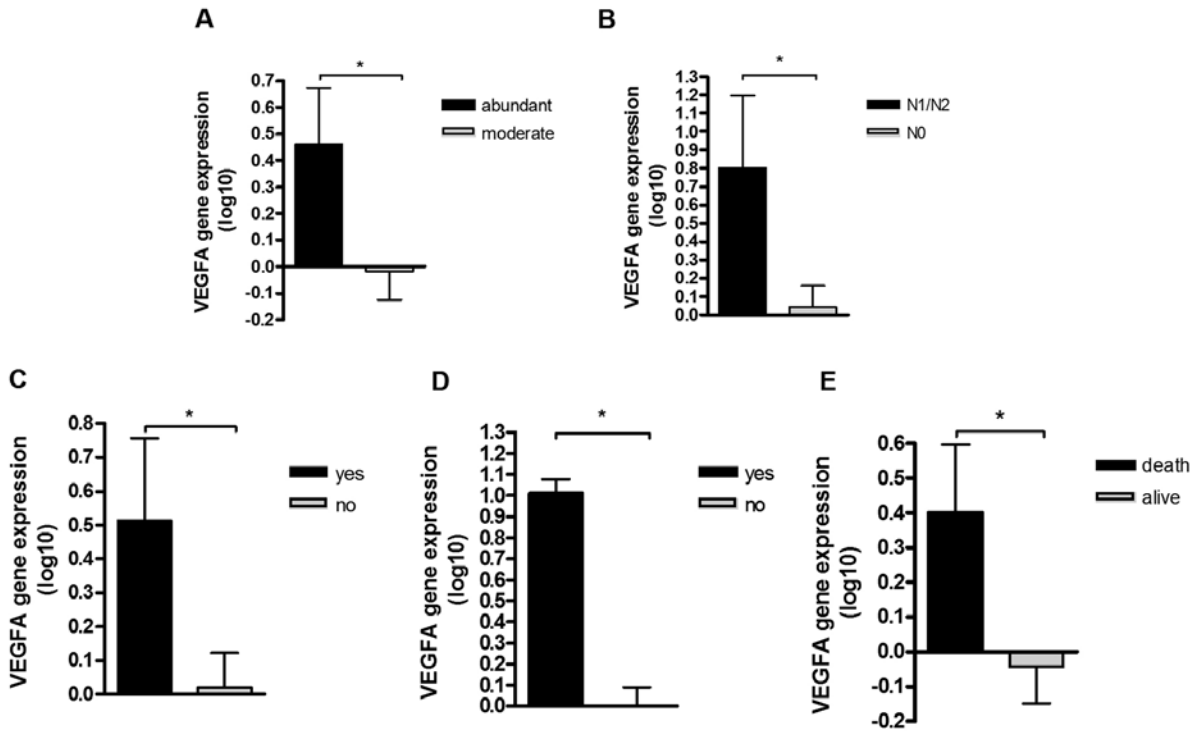

Figure 2. Quantitative gene expression of VEGFA in canine mammary tumors. Correlation between (A) tumor vascularization (*p=0.03), (B) lymph-node involvement (*p=0.02), (C) metastasis (*p=0.04), (D) tumor recurrence (*p=0.0002) and (E) censorship (*p=0.04). Value of gene expression in log10. *Significant differences were identified using the Student's t-test.

was significantly overexpressed in tumors with moderate vascularization (p=0.02; Fig. 3A), without metastasis (p=0.01; Fig. 3B), without recurrence (p=0.02; Fig. 3C) and female dogs that were still alive when the study was terminated (p=0.01; Fig. 3D).

Discussion

In this study, we have demonstrated the prognostic value of VEGF and HIF-1α on female dogs with malignant mammary tumors and found a correlation between an increase of VEGF serum levels and clinicopathological parameters of worse prognosis, which decreased the survival rate in female dogs with malignant mammary tumors compared to the control group. Additionally, there was a significant increase of VEGF serum concentration in female dogs with malignant mammary tumors compared to the control group.

Serum tumor markers play an important role in the early diagnostic, prognostic determination, specific therapeutic response prediction, precocious detection of tumor recurrence after surgery, and follow-up in advanced disease therapy (43,44). In this context, the VEGF is considered an important indicator of the development of cancer, and its serum levels can be used to estimate tumor progression (45).

In accordance with our results, some studies in humans have shown that serum VEGF levels were higher in patients with breast cancer when compared to healthy subjects and inversely correlated with survival (46,47). On the other hand, Duranyildiz *et al* (48) and El Tarhouny *et al* (49) did not observe any significant difference between serum VEGF levels in patients with breast cancer and the control group.

VEGF signaling contribute to the biology and clinical behavior of canine mammary carcinomas (4). By considering canine mammary tumors, Kato *et al* (30) found higher serum VEGF levels in female dogs with malignant tumors compared to female dogs with benign tumors, as well as in female dogs that had lung metastasis after tumor excision. Marked VEGF mitogenic properties led to an increase in the permeability of blood vessels, allowing cancer cells to pass through to extra-vascular spaces and form distant metastases (50). Significantly higher serum VEGF concentrations in metastatic and invasive tumor patients with breast cancer when compared to patients with non-metastatic and non-invasive tumors have been previously reported (51,52).

The hypoxic environment, which induces gene expression changes and biological features leading to poor outcomes, is important in the modulation of tumor angiogenesis (53,54). In this regard, it has been shown that HIF-1 is a leading regulator of tumor angiogenesis following hypoxia, which has been demonstrated to be significantly associated with the morbidity and mortality of breast cancer (55,56). However, to the best of our knowledge, there have been no studies investigating the quantification of HIF-1α in the serum of female dogs bearing malignant mammary neoplasias in the literature. In humans, there was a study that compared the serum levels of HIF-1α in diabetic patients with breast cancer and control groups, demonstrating the HIF-1α levels were markedly higher in the patient group than in the controls (55). Other authors found higher serum HIF-1α levels in patients with lung cancer (57), as well as patients with liver cancer (46).

As expected, the *VEGFA* gene was overexpressed in female dogs with worse prognosis, however, *HIF-1α* was

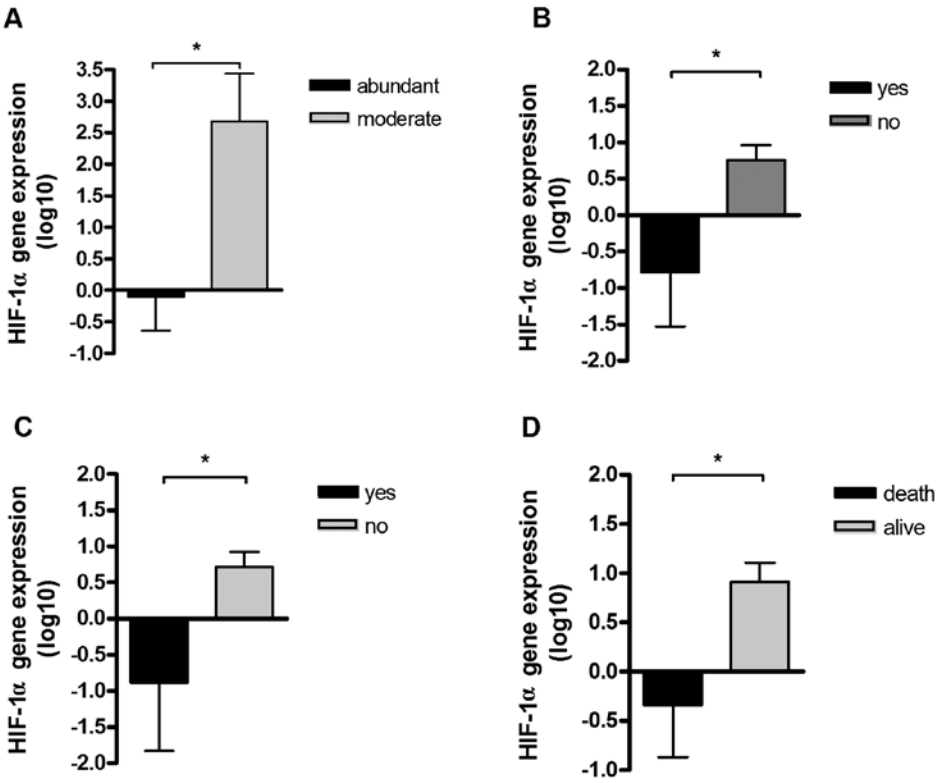

Figure 3. Quantitative gene expression of HIF-1α in canine mammary tumors. Correlation between (A) tumor vascularization (*p=0.02), (B) metastasis (*p=0.01), (C) tumor recurrence (*p=0.02) and (D) censorship (*p=0.01). Value of gene expression in log10. *Significant difference was identified using the Student's t-test.

overexpressed in female dogs with better prognosis. Recent advances in cancer research have indicated that in hypoxia conditions, the hypoxia-inducible factors, such as HIF-1α, may activate the transcription of several genes that play key roles in many critical aspects of cancer biology, particularly *VEGF* (15,16,25-28). However, in our study, the gene expression of *HIF-1α* did not follow the same pattern as the *VEGFA* gene expression, and this can be explained by the fact that *HIF-1α* acts synergistically with numerous factors and oncogenes that regulate the expression of *VEGF* (56,58) suggesting other, as yet unidentified factors that promote *VEGF* gene transcription by binding to hypoxia response elements (HREs) (59).

In agreement with our results, findings of previous studies associated the increase of *VEGF* expression with neoplastic cell aggression, disease dissemination and tumor mass development, reducing survival rate of patients with breast cancer (9,33,34,60,61). In dogs, the expression of *VEGFA* was previously studied by qPCR and it was associated with tumor aggression (37). Kallergi *et al* (25) analyzed VEGF expression in metastatic and non-metastatic mammary cell lines and found an increase of VEGF expression in the more aggressive cells, especially metastatic ones.

An increased expression of *HIF-1A* in various carcinomas has been associated with aggressive behavior, enhanced rates of distant metastases, decreased survival rates and increased resistance to the treatment of breast cancer in patients, as opposed to our results (62). Results showing a correlation of the role of HIF-1α in cancer progression in the literature are controversial, as it is known that HIF-1α expression is increased with tumor growth because larger tumors are generally more hypoxic than smaller ones (63). However, depending on the severity of hypoxic stimulus, HIF-1α serves as a pro-death gene capable of promoting apoptosis and cell death (43). Under conditions of severe hypoxia cells seem to survive, initiating a cascade of events leading to apoptosis and cell death, thus leading to a reduction in tumor progression (42).

Despite the fact that our investigations have not confirmed the correlation between HIF-1α and clinicopathological characteristics, our results showed that VEGF is correlated with characteristics of poor prognosis. Therefore, in view of our own observations and conflicting opinions reported in other studies, it seems that only VEGF can be employed as a potential prognostic marker for routine use in the clinic, as it is useful in predicting disease progression and tumor recurrence in female dogs with malignant mammary tumors.

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