PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(3):210-219, March 2020
DOI: 10.1590/1678-5150-PVB-6489

Original Article
Small Animal Diseases

Osteopontin expression and its relationship with prognostic biomarkers in canine mammary carcinomas[1]

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ABSTRACT.- Monteiro L.N., Salgado B.S., Oliveira D.E., Rivera-Calderon L.G, Montoya-Flórez L., Sanctis P. & Rocha N.S. 2020. **Osteopontin expression and its relationship with prognostic biomarkers in canine mammary carcinomas.** *Pesquisa Veterinária Brasileira 40(3)210-219* Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Dr. Valter Maurício Corrêa s/n, Botucatu, SP 18618-681, Brazil. E-mail: maomontoya53@yahoo.es, lmontoyaf@unal.edu.co

Osteopontin is a glycophosphoprotein implicated in different physiologic and pathologic processes and is known to be involved in progression and metastasis of various cancers in humans, but this relation is still little explored in the veterinary. The aim was to evaluate the expression of osteopontin in canine mammary carcinomas and its relation with well-established canine mammary tumor biomarkers. For that, expression of OPN, EGFR, HER2, and c-Kit were evaluated along with Ki67 rate in 43 mammary carcinomas. Osteopontin was demonstrated to be expressed by neoplastic epithelial cells in all carcinomas as well as in stromal cells from the tumor microenvironment. Relation between high osteopontin expression and EGFR positivity (P<0.001) and HER2 overexpression (P=0.012) was demonstrated. In conclusion, high OPN expression seems to be related to poor prognosis and MAPK pathway activation, given the association with EGFR and HER2, members of the MAPK signaling pathway.

INDEX TERMS: Dogs, cancer, osteopontin expression, prognostic biomarkers, canine mammary carcinomas, immunohistochemistry, epidermal growth factor receptor, human epidermal growth factor receptor 2.

RESUMO.- [**Expessão de osteopontina e sua relação com biomarcadores prognóstico nos carcinomas mamários caninos.**] A osteopontina é uma glicofosfoproteina implicada em diferentes processos fisiológicos e patológicos, sendo conhecida por estar envolvida na progressão e metástase de vários cânceres nos humanos, no entanto, essa relação é ainda pouco explorada na veterinária. O objetivo deste trabalho foi avaliar a expressão da osteopontina nos carcinomas mamários caninos e sua relação com biomarcadores bem estabelecidos para esta neoplasia. Para isto, foi avaliada a expressão de OPN, EGRH, HER2 e c-Kit juntamente com a taxa de Ki67 em 43 carcinomas mamários. A osteopontina foi expressa pelas células epiteliais neoplásicas em todos os carcinomas, assim como, nas células estromais do microambiente tumoral. Foi demonstrada uma relação entre uma alta expressão de osteopontina e positividade para EGFR (P<0.001) e superexpressão de HER2 (P=0.012). Em conclusão, alta expressão de OPN parece estar relacionada com mau prognóstico e ativação da via MAPK, devido a sua associação com EGRF e HER2, os quais são membros desta via de sinalização.

TERMOS DE INDEXAÇÃO: Cães, câncer, osteopontina, biomarcadores, carcinomas mamários caninos, imunohistoquímica, receptor de fator de crescimento epidérmico, receptor de fator de crescimento epidérmico humano 2.

[1] Received on August 23, 2019
Accepted for publication on September 29, 2019.

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INTRODUCTION

Several studies have pointed cancer as the main cause of death for dogs in developed countries and 45% of dogs have over 10 years of age (Bronson 1982, Michell 1999, Proschowsky et al. 2003, Battisti et al. 2013, Dobson 2013, Daleck et al. 2016). In Brazil, the disease figures as the second most common cause of death for dogs (Fighera et al. 2008, Trapp et al. 2010, Andrade et al. 2012, Battisti et al. 2013, Daleck et al. 2016) and it is estimated that one in five dogs will develop cancer. The skin and subcutaneous tissue being the most prevalent followed by mammary, hematopoietic and bone tumors. Mammary tumors are the most common cancer diagnosed in women, likewise in female dogs (Dobson 2013, Pawlowski et al. 2013, Salas et al. 2015, Dias et al. 2016, Salas et al. 2016). Statistical surveys estimate that mammary neoplasms represent about 50% of all tumors afflicting female dogs, of which at least 40% are malignant (Brodey et al. 1983, Andrade et al. 2012, Feliciano et al. 2012, Battisti et al. 2013, De Nardi et al. 2013, Li et al. 2013, Pawlowski et al. 2013, Santos et al. 2013, Peña et al. 2014, Arias et al. 2015, Salas et al. 2015, Dias et al. 2016, Salas et al. 2016, Soler et al. 2016).

Advances in the diagnosis and therapy of the animals, the application of efficient measures in the prevention of infectious diseases through vaccination and deworming, in addition to improvements in the nutritional quality of dog food, have contributed towards a higher quality of life and longevity for dog (Dobson 2013, Salas et al. 2015), resulting in an increase in the diagnoses of neoplasms for the species (Pawlowski et al. 2013, Santos et al. 2013, 2014, Soler et al. 2016). Canine mammary tumours are highly heterogeneous in morphology and behavior and successful clinical management requires robust prognostic factors. The biological behavior of canine mammary neoplasms is widely variable in morphology and behavior, making validation and the use of tumor biomarkers to support the diagnosis and prognosis extremely important to successful clinical management (Graham & Myers 1999, Kandioler-Eckersberger et al. 2000, Arias et al. 2015, Damasceno et al. 2016b, Psyrri et al. 2017). Immunohistochemistry techniques may be useful to anticipate a diagnosis of cancer and to present prognostic information regarding the disease (Graham & Myers 1999, Kandioler-Eckersberger et al. 2000, Zacchetti et al. 2003, Peña et al. 2014, Santos et al. 2014, Arias et al. 2015, Damasceno et al. 2016a, Soler et al. 2016). Several biomarkers have been identified and associated with the survival rates of dogs afflicted with mammary neoplasms, such as the estrogen and progesterone hormone receptors (Sartin et al. 1992, Nieto et al. 2000), p53 (Lee et al. 2004) e-cadherin (Marmor et al. 2004), caspase-3 (West et al. 2008), cathepsin D (Lemmon & Schlessinger 2010) survivin (Bongiovanni et al. 2015), cell proliferation markers such as Ki-67 and PCNA (Zacchetti et al. 2003) epidermal growth factor receptor (EGFR) (Nieto et al. 2000, Rangaswami et al. 2006, Vollmann-Zwerenz et al. 2010, Arias et al. 2015, Elebro et al. 2016) and human epidermal growth factor receptor 2 (HER-2) (Sartin et al. 1992, Zacchetti et al. 2003, Carvalho et al. 2013, Ferreira et al. 2014, Peña et al. 2014, Silva et al. 2014, Burrai et al. 2015, Theocharis et al. 2015, Damasenco et al. 2016a, 2016b).

The receptors tyrosine kinase (RTK) of the ErbB family, known as EGFR/HER-1, erbB-2/HER-2, erbB-3/HER-3 and erbB-4/HER-4, play an important molecular control role as a signal for the development and maintenance of several organs and systems (Graham & Myers 1999, Nieto et al. 2000, Lee et al. 2004, Marmor et al. 2004, West et al. 2008, Damasceno et al. 2016b) since they are a group of primary mediators for the fundamental cell responses (Lemmon & Schlessinger 2010). Furthermore, it has been shown to have an important role to contribute to a better understanding of the progression mechanisms in malignant mammary tumors (Graham & Myers 1999, Bongiovanni et al. 2015, Damasceno et al. 2016b). Its role appears to be associated with increased angiogenesis and metastasis (Bongiovanni et al. 2015). In addition, another receptor tyrosine kinase (c-Kit) also plays an important role in cell proliferation and differentiation (Liang et al. 2013). These receptors have been widely studied due to recent discoveries regarding their involvement in the pathogenesis of hyperproliferative diseases such as cancer (Vollmann-Zwerenz et al. 2010, Liang et al. 2013, Elebro et al. 2016).

Recently, osteopontin (OPN), an adhesive glycophosphoprotein found in tissues and body fluids that is involved in both physiological and pathological processes (Rangaswami et al. 2006, Vollmann-Zwerenz et al. 2010, Liang et al. 2013, Shevde & Samant 2014, Elebro et al. 2016) has been widely pointed as a biomarker with potential prognostic implications for cancer due to its functional role over the tumor progression and metastasis control pathways (Rangaswami et al. 2006, Weber et al. 2010, Anborgh et al. 2011, Shevde & Samant 2014, Burrai et al. 2015, Damasceno et al. 2016a, Li et al. 2016, Psyrri et al. 2017, Wei et al. 2017). OPN belongs to the family of small integrin-binding glycoproteins related to N playing a key role in cell-matrix and cell-cell communication and interaction, modulating cellular behavior through autocrine and paracrine mechanisms (Fisher et al. 2001, Bellahcène et al. 2008). Studies have described its role in several development and differentiation processes in tissues, including bone (Yamate et al. 1997), skin (Chang et al. 2008) and mammary glands (Rittling & Novick 1997, Carvalho et al. 2013, Silva et al. 2014, Psyrri et al. 2017). In addition, it plays a key role in immune and inflammatory responses (Tuck & Chambers 2001, Rangaswami et al. 2006, Rittling & Singh 2015), including the healing process of wounds (Liaw et al. 1998).

The expression of OPN is up-regulated by many factors such as epidermal growth factor (EGF), transforming growth factor-Beta (TGF-β), tumor necrosis factor α (TNFα), interferon gamma (IFN-γ) and interleukin-1 β (IL-1β) (Rangaswami et al. 2006). Regarding pathological events, studies have shown that OPN is overexpressed in sepsis (Hirano et al. 2015), autoimmune diseases, cardiovascular diseases (Waller et al. 2010), neurodegenerative diseases (Carecchio & Comi 2011) and several tumors, especially carcinomas (Coppola et al. 2004, Anborgh et al. 2011, Shevde & Samant 2014). In the case of tumors, OPN's potential for predicting the prognosis was initially reported by Chambers et al. (1996), later, Tuck et al. (1997) have described the relationship between OPN overexpression and tumor progression in human mammary neoplasms, suggesting that OPN could be employed as a tumor prognostic marker both in tumor cells and in plasma.

There are few studies in the field of canine immunohistochemistry assessing the role of biomarkers in general, and OPN specifically, in tumor initiation and progression, as well as in the identification of patients with high disease recurrence risks. This study has analyzed the immunoexpression of OPN, EGFR, HER2, c-Kit

and Ki67 in *ex vivo* canine mammary carcinomas to obtain data supporting a better understanding of the role OPN plays and its relationship with other established biomarkers for this particular tumor.

MATERIALS AND METHODS

Tissue samples. The Brazilian Ethics Commission for the Use of Animals (protocol no. 88/2011 - CEUA) approved the study and the owners of all animals involved in the study have signed a Free and Clarified Consent Term authorizing the collection of material and the use of the data in research papers. Canine mammary carcinomas specimens (*n*=43) were collected at the time of surgical excision by research of the Investigative and Comparative Pathology Laboratory, "Universidade Estadual de São Paulo", Botucatu, Brazil, and it were used for the study. Samples were fixed in 10% neutral formalin and embedded in paraffin wax. Sections (4µm thick) were obtained and stained with hematoxylin and eosin for histological examination in order to confirm the diagnosis of mammary carcinoma. Tumor classification was defined according to the WHO classification of canine mammary tumors (Misdorp et al. 1999). Tumor sections were examined in an optical microscope (Zeiss® Axio Lab. A1) by three independent pathologists. For each slide, 10 fields were read with 400x magnification. Inter-observer variation was resolved by simultaneous re-evaluation.

Immunohistochemistry. For the immunohistochemistry assays, 3µm thick sections were obtained from paraffinized tissue blocks and subsequently deparaffinizated and rehydrated. The primary antibodies used in this study are summarized in Table 1. A polymer-based labeling system kit (NovoLink Polymer System, Novocastra Laboratories, Newcastle, UK) was used for detecting the antigen-antibody reaction and peroxidase and protein blockages. Antigen retrieval was carried out by heat treatment in 10mM citrate buffer, pH 6.0. After cooling (20 minutes at room temperature), sections were sequentially immersed in solutions provided in the kit according to manufacturer's instructions in order to block the endogenous activity of peroxidase and unspecific proteins. The slides were incubated overnight at 4°C with the specific antibodies. Subsequently, 3,3' diaminobenzidine (DAB) tetrahydrochloride was used as a chromogen in order to allow the visualization of antigen-antibody reaction. The slides were then counterstained using Harris's hematoxylin, dehydrated, and mounted for microscopic assessment.

Immunohistochemical results evaluation. The evaluation of the immunohistochemistry results was performed by three pathologists. OPN was considered positive whenever cytoplasmic staining was observed in the neoplastic and stromal cells. The assessment of OPN expression in neoplastic cells was performed semi-quantitatively using the Allred 8-unit system (Allred et al. 1993). In this scoring system, the tumor epithelial cells proportion score and intensity score were determined for each tumor, represented by one slide. The proportion score included the fraction of positively stained tumor cells and was as follows: 0 = none; 1 = <1/ 100th; 2 = 1/100th to 1/10th; 3 = 1/10th to 1/3; 4 = 1/3 to 2/3; 5 = >2/3. The estimated average staining intensity of positive tumor cells was expressed as follows: 0 = none; 1 = weak; 2 = intermediate; 3 = strong. For statistical purposes, an OPN score of 1–3 was considered low (+1), an OPN score of 4-6 was considered intermediate (+2), and an OPN score of 7-8 was considered high (+3). The expression of OPN was also evaluated in peritumoral inflammatory cells and tumor stromal cells.

The expression of HER2 was evaluated according to the Dako Cytomation Hercep Test scoring system: 0 = no staining or membrane staining in less than 10% of tumor cells; 1+ = faint, barely perceptible membrane staining in more than 10% of tumor cells, the cells are stained only in part of the membrane; 2+ = weak to moderate, complete membrane staining observed in more than 10% of tumor cells; and 3+ = strong, complete membrane staining in more than 10% of tumor cells. Cases were considered positive (overexpressed) for HER2 when immunostaining was characterized as 2+ or 3+. EGFR staining was classified in positive or negative, according to Dako's EGFR pharm Dx interpretation manual.

For Ki67, four categories were defined as follows: <10%, 10-25%, 26-50% and >50% of stained nuclei. For c-kit expression, the reaction product was evaluated along the cell membrane and in the cytoplasm. The quantity of immunoreactive cells was estimated according to the classification adopted by Biermann et al. (2007) none; <10%; 10–75%; and >75% of the cells. The level of immunoreactivity was assessed based on its predominant intensity: weak (+); moderate (++); and strong (+++).

The final immunoreactivity score was calculated as strong (4), when at least 75% of cells exhibited at least moderate immunoreactivity; in cases of weak immunoreactivity in <10% of all tumor cells, the final score was considered as (1). The score (0) was considered negative. The score (3) was assigned if weak immunoreactivity was present in >75% of tumor cells or if strong or moderate staining was observed in 10-75% tumor cells. All other cases were given a score of (2).

Positive and negative controls were included in each run in order to guarantee the reliability of the assays. For OPN, canine kidney was used. Additionally, a canine mammary carcinoma already recognized as HER-2 positive, a canine cutaneous mast cell tumor positive for c-Kit, and a canine cutaneous squamous cell carcinoma recognized as positive for EGFR and Ki67 were used. Rabbit and mouse IgGs (Dako, Carpinteria/CA) were used in tumor samples for negative control purposes.

Statistical analysis. Differences in the expression of proteins were compared using Fisher's exact test or Pearson's X2 test for qualitative variables. All statistical tests were two sided, and statistical significance was accepted at $P<0.05$. All analyzes were performed using the Prism GraphPad software version 5.0 (San Diego/CA).

Table 1. Antibodies used in the immunohistochemical study

Antibody	Clone	Dilution	Origin	Source
Osteopontin	LFMb-14	1:50	Novocastra Laboratories, UK	Mouse
Ki67	MIB1	1:50	Dako	Mouse
CD117	104D2	1:400	Dako	Mouse
HER-2	policlonal	1:2000	Dako	Rabbit
EGFR	NCL-EGFR	1:100	Novocastra Laboratories, UK	Mouse

RESULTS

Immunohistochemistry

Cytoplasmic OPN staining was observed consistently in neoplastic cells of all mammary tumors evaluated (43/43), always presenting an intermediate or high classification score (Fig.1); 12 of 43 (28%) samples presented intermediate score and 31 of 43 (72%) presented high score. We observed that all samples presented more than 50% of positive neoplastic cells and presented a high score variation due to the staining intensity. OPN positivity was observed mainly within the

cytoplasm, sometimes perinuclearly or, less commonly, in a cell surface distribution in the neoplastic cells. No sample was immunohistochemically negative for osteopontin.

Strong OPN expression was observed consistently in the epithelial component and in the myoepithelial cell layer, with cells presenting a cytoplasmic expression pattern. The surrounding stroma was usually OPN positive, since the OPN immunoexpression was also observed in the tumor stromal matrix component, stromal fibroblasts, vascular endothelial cells, muscular cells, macrophages and other inflammatory cells in all samples (Fig.2). Consistent OPN immunoexpression was also observed in areas with necrosis (Fig.3), chronic inflammation (Fig.4) and on invasive tumor borders (Fig.5). In addition, the weak OPN immunoexpression was observed in luminal mammary cells from the normal mammary tissue adjacent to neoplastic *foci* (Fig.5).

Relationship between osteopontin and other markers

From the 43 samples of mammary carcinoma evaluated, 34 (79.07%) were positive for HER2 and 36 (83.72%) were positive for EGFR. Additionally, 28 tumors with OPN high expression (90.3%) also presented HER2 overexpression, revealing a statistically significant association (P=0.012). For EGFR, 29 (93.5%) of the positive cases also presented high OPN expression, revealing a statistically significant relationship (P<0.001). All cases (43/43) were positive for c-Kit, presenting score variation between 1 and 4. There was no significant association with c-Kit (P>0.05). Likewise, 27 from the 31 cases with OPN overexpression (87%) revealed less than 25% neoplastic cells positive for Ki67. However, no statistically significant relationship could be noted between OPN and Ki67.

DISCUSSION

This study aimed at determining the expression level of the glycoprotein OPN in the stroma and neoplastic cells of canine mammary tumors, as well as its association with others proteins by means of immunohistochemistry assays. In humans, there are well documented reports showing the overexpression of OPN in malignant tissues and in the plasma, as well as the correlation with the tumor stage in the brain (Zakaria et al. 2016), mouth, esophagus, stomach, large intestine, liver,

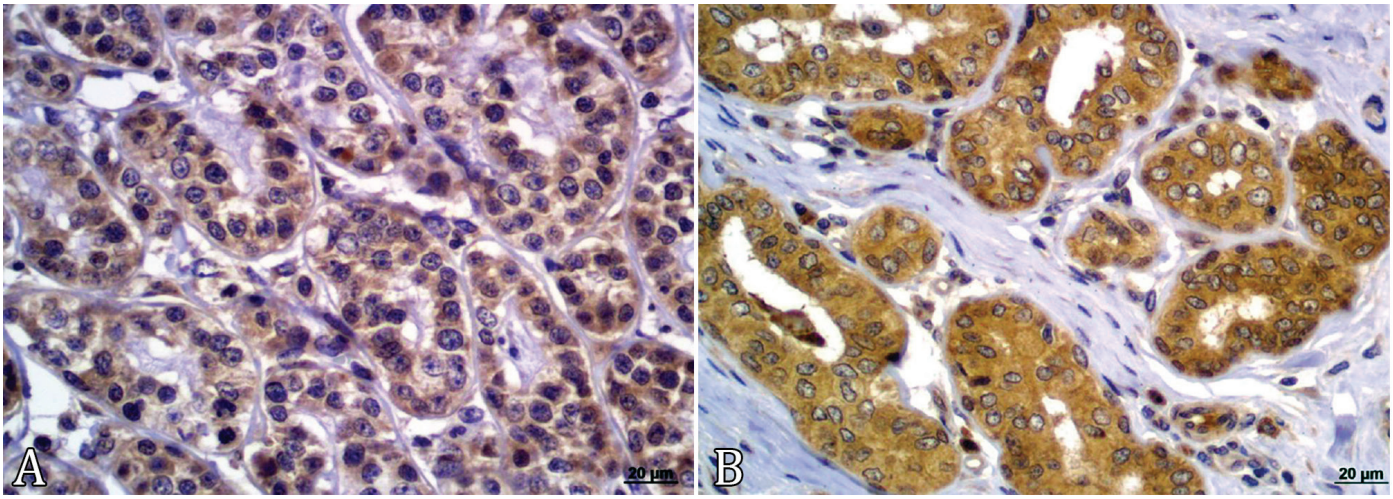

Fig.1. Osteopontin immunoexpression in canine mammary neoplasms. (**A**) Intermediate osteopontin immunoexpression, (**B**) high osteopontin immunoexpression. DAB immunohistochemistry, Harris hematoxylin counterstain, bar = 20µm.

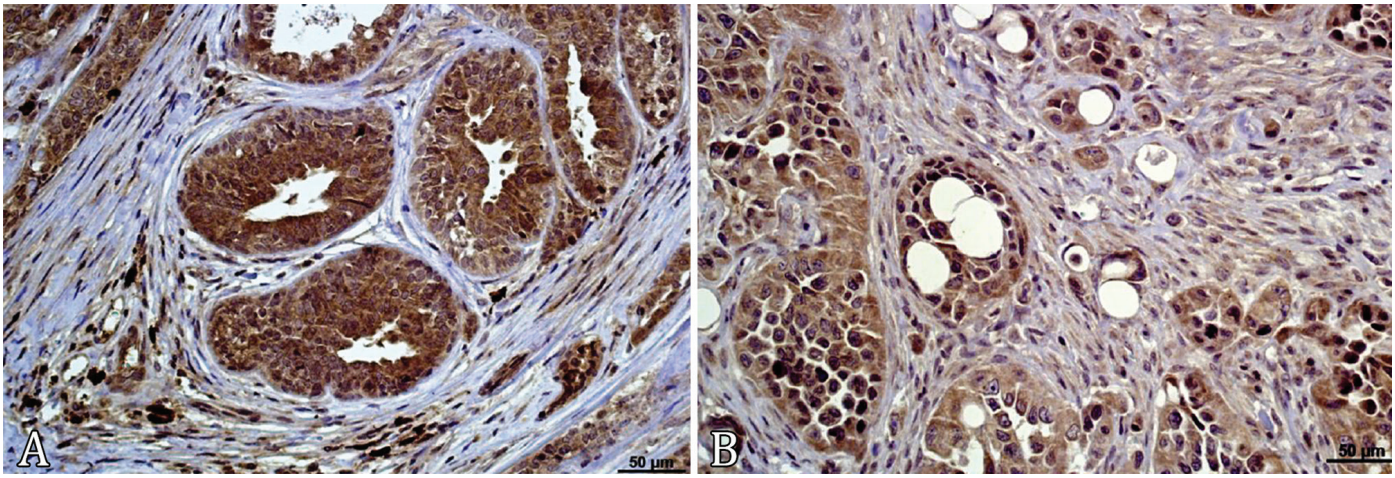

Fig.2. (**A**-**B**) Osteopontin-positive stromal cells, including inflammatory cells, can be observed within the neoplastic microenvironment. DAB immunohistochemistry. Harris hematoxylin counterstain, bar = 50µm.

pancreas, kidney (Coppola et al. 2004), ovaries, prostate (Tilli et al. 2014), lungs (Yan et al. 2015) and breasts (Bramwell et al. 2014). These studies clearly point towards the importance of this glycoprotein and its multidimensional ability to influence biological events associated with tumorigenesis and tumor progression, including the possibility of employing it as a molecular parameter in the prognostic evaluation of cancer patients. Since there are no similar reported studies in dogs, a clarification regarding its significance in canine mammary tumors was required. OPN-producing cells and OPN deposition in extracellular matrix were previously identified through IHC in canine bone and cartilage (Schnapper & Meyer 2004). Additionally, a quantitative real-time reverse transcription polymerase chain reaction array was established to quantify the expression levels of 49 genes relevant to carcinogenesis in laser-microdissected tumor cells of 10 benign and 13 metastatic canine mammary tumors (Klopfleisch et al. 2010). The study in question detected OPN in normal tissues, but observed no significant differences in the expression levels of osteopontin among any of the groups tested.

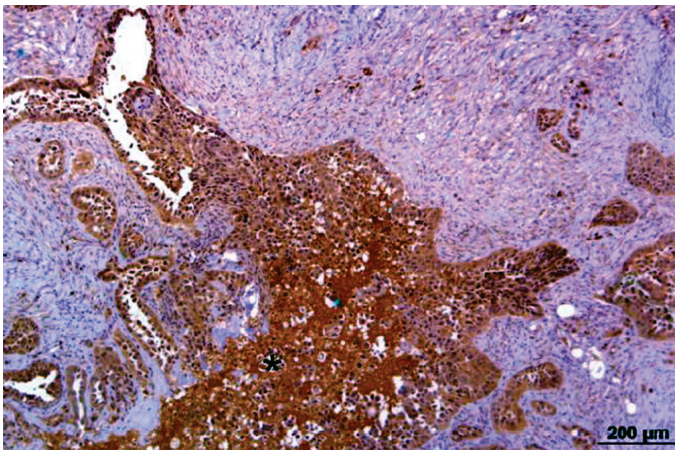

Fig.3. More intense OPN expression can be observed in necrotic areas (asterisk). DAB immunohistochemistry. Harris hematoxylin counterstain, bar = 200µm.

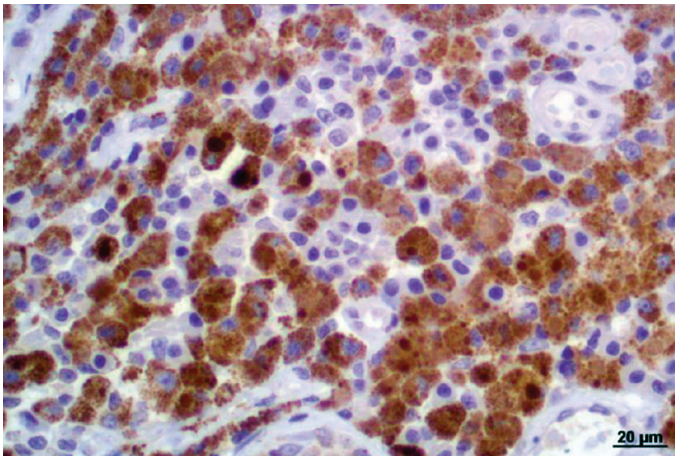

Fig.4. Osteopontin-positive macrophages. DAB immunohistochemistry. Harris hematoxylin counterstain, bar = 20µm.

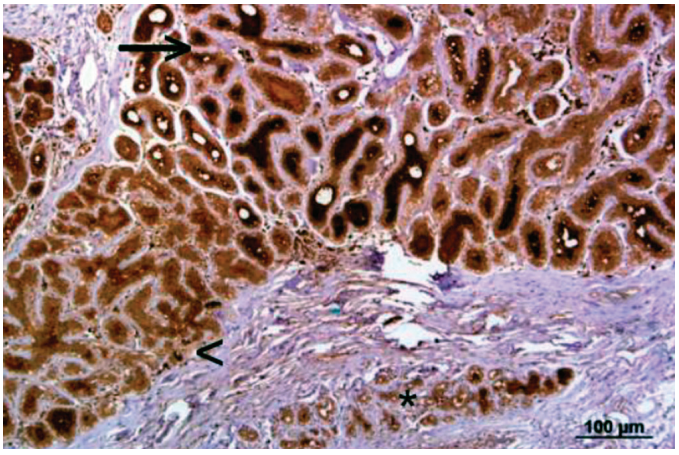

Fig.5. Osteopontin expression in normal (asterisk) tissue, neoplastic *foci* (arrows) and invasive tumors borders (arrowhead). DAB immunohistochemistry. Harris hematoxylin counterstain, bar = 100µm..

Presently, the scientific community fully accepts that OPN is expressed by tumor cells both in humans and animals and that it affects the malignant properties of neoplastic cells, specifically by affecting their ability to grow, invade, and metastasize. In addition, it is also known that OPN is expressed in both normal and malignant tissues (Shevde & Samant 2014, Ng et al. 2015) and effectively mediates many physiological and pathological events (Kahles et al. 2014).

In this study, we have found that all carcinomas were stained positively for OPN and, according to the immunohistochemical grading system employed; tumors presented consistently highly expression levels for the protein. This finding is in accordance to studies that focused on OPN levels in women breast tumor tissue (Tuck & Chambers 2001, Coppola et al. 2004, Rodrigues et al. 2009, Bramwell et al. 2014), since authors report a high frequency of OPN-positive samples. For example, some authors reported a positivity of 88.4% for OPN in metastatic human brain tumors (Zakaria et al. 2016) and a positivity of 100% in human hepatocellular carcinomas (Tsai et al. 2012) similarly to what was observed in this study. Although canine mammary tumors may occasionally be histologically and biologically different than human breast cancer, the results regarding OPN immunoexpression seems to be in concordance. This study and that of others (Tuck & Chambers 2001, Rodrigues et al. 2009, Luo et al. 2011) reported that OPN is expressed both in the epithelial and stromal components of neoplastic wounds. In humans, OPN immunoexpression is also observed outside neoplastic cells and reveals a variable staining pattern in which luminal cells from normal mammary tissue, luminal tumor cells, stromal fibroblasts, macrophages, lymphocytes, and blood vessels are weakly to highly stained (Kim et al. 1998, Tuck et al. 1998, Tuck & Chambers 2001, Rodrigues et al. 2009, Luo et al. 2011).

In this study, it was observed that cells from the tumor microenvironment and cells adjacent to the tumor presented OPN positivity in all samples. Studies have shown that, aside from being expressed in different cell types, including immune system cells, the expression of OPN is highly increased during the inflammatory process (Rittling & Singh 2015) and regulated positively through several growth factors and cytokines, including LPS, Ang II, NO, IL-1b, IL-2, IL-3, IFN-g, TNF-α and TGF-β (Denhardt et al. 2003). In our study, we observed a more intense OPN immunoexpression in cells near necrotic or inflammatory *foci*, as well as in invasive tumor areas, in

agreement with others reports (Brown et al. 1994, Hirota et al. 1995, Tuck et al. 1998). These findings suggest that those cell types may contribute to OPN production levels. However, our understanding regarding the molecular mechanisms involved in the regulation of OPN expression remains incomplete (Kahles et al. 2014). The presence of OPN in the tumor stroma and on the surface of tumor cells interfacing with the stroma suggests that this glycoprotein may participate in adhesive interactions at the tumor/normal tissue interface. Studies have shown that OPN overexpression, especially the OPNb and OPNc variants, at esophageal adenocarcinoma enhances tumor cell invasion and metastasis (Lin et al. 2015) and also contribute towards macrophage adhesion and migration (Brown et al. 1994).

In a previously reported study (Rodrigues et al. 2009) using invasive human breast cancer cases, no statistically significant association was reported between stromal OPN expression, major clinical and pathological parameters, and some of the most commonly used molecular markers for those tumors. Whether epithelial and stromal OPN has distinct roles during neoplastic development and progression is an important question to be further addressed, but it seems to be related to metastasis in various neoplasms (Brown et al. 1994).

In this study, different tumor markers were tested for their association with OPN in canine mammary carcinomas, but only EGFR and HER2 showed a statistically significant relationship with OPN-positive immunostaining scores. These components are part of the MAPK signaling pathway, which is recognized as an important pathway for carcinogenesis, particularly for the epithelia (Sebolt-Leopold & Herrera 2004). The findings suggest an osteopontin-associated activation of the MAPK pathway in canine mammary neoplasms, which is in agreement with the findings presented by other authors (Brown et al. 1994, Frey et al. 2007), revealing a possible relationship between OPN and the MAPK pathway in breast cancer (Tuck et al. 2003, Rodrigues et al. 2009), lung adenocarcinomas (Frey et al. 2007), hepatocellular carcinoma (Tsai et al. 2012) and in actinic keratosis/cutaneous squamous cell carcinomas (Luo et al. 2011) in humans. However, this finding was reported to be absent in other tumor histotypes such as mesothelioma (Frey et al. 2007), suggesting that this alteration may not be a distinctive feature for all tumor types.

In dogs, the expression of the erbB family components was previously evaluated in canine mammary tumors. A relatively high expression of the *ERBB1* and *ERBB2* genes suggests an important contribution to carcinogenesis in canine mammary tumors (Matsuyama et al. 2001, Singer et al. 2012). The overexpression of tyrosine kinase receptors EGFR and HER2 - proteins derived from *ERBB1* and *ERBB2* genes, respectively - is observed in many human cancers including bladder, breast, colon, and lung cancers (Eccles et al. 1995). HER2 overexpression is usually associated with poor prognosis indicators in canine mammary tumors such as tumor size, high histological grade, invasion, and high proliferation rates (De las Mulas et al. 2003).

Regarding the relationship between HER2 and OPN, Rodrigues et al. (2009) also did not find any association in women. However, this association was statistically significant in the tumors studied by our group. This finding may indicate the existence of a co-regulator expressed differently in human and canine mammary neoplasms, leading to the activation of this specific RTK in the canine counterpart.

In this study, 83.72% of the samples were positive for EGFR, a protein previously described to be related to reduced cure and overall survival rates in canine mammary tumors (Gama et al. 2009). We have also identified a statistically significant relationship between high OPN expression and EGFR positivity, which we believe to be a synergistic and complementary relationship between the molecules, in accordance to what was reported by other authors in human breast cancer (Tuck et al. 2003, Rodrigues et al. 2009) and hepatocellular carcinomas (Tsai et al. 2012).

For the latter, higher OPN and EGFR expression were significantly associated with advanced histological grades, advanced pathological stages, and poor survival rates (Hirota et al. 1995). Cell migration regulated by OPN is said to be dependent on the epidermal growth factor (EGF) and hepatocyte growth factor (HGF). OPN induces EGF receptor (EGFR) mRNA expression, EGFR tyrosine kinase activity, HGF receptor (Met) mRNA and protein expression, as well as increasing Met kinase activity during tumor cell migration in human mammary cancer cell lines (Tuck et al. 2003).

Previous reports indicated that ligation of OPN with integrin leads to c-Src-dependent transactivation of EGFR, resulting in the activation of downstream signaling pathways, including PI3-k, Ras–MAPK, phospholipase C, and protein kinase C (PKC) in cancer cells (Tuck et al. 2003). The transformation of epithelial cells induced by tissue-specific overexpression of EGFR *in vivo* provides direct evidence of the role EGFR plays in carcinogenesis (Yarden & Sliwkowski 2001).

These features which indicate a relationship between EGFR/HER2 and OPN overexpression are interesting since an arsenal of antibodies and tyrosine kinase inhibitors for growth factor receptors targeted to the MAPK pathway are currently either in development or already in clinical use, consequently being expected to be effective against tumors overexpressing OPN. Various therapeutic agents directed against EGFR and HER2 have provided promising alternatives to traditional chemotherapy in the search for better treatments for cancers overexpressing these tyrosine kinase receptors (Kamath & Boulamwini 2006). This could represent an evolution in the conventional treatment of canine mammary tumors focused in highly OPN expressing neoplasms.

CONCLUSION

Osteopontin overexpression is related to EGFR and HER2 expression in canine mammary tumors, probably by the activation of the MAPK signaling pathway. Although the mechanisms involving OPN and the progression of canine mammary carcinomas remain unknown, this study suggests that OPN, EGFR, and HER2 play important roles in canine mammary tumors carcinogenesis. These findings raise the question of whether is possible to use specific drugs to block the signaling pathway in OPN overexpressing tumors.

Conflict of interest. - The authors do not have any conflicts of interest to declare.

Acknowledgements. - This study was supported by "Fundação de Amparo à Pesquisa do Estado de São Paulo" (FAPESP), Brazil research grant 2010/51596-2 and by "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq), Brazil, research grant 479178/2010-0.

REFERENCES

Allred D.C., Clark G.M., Elledge R., Fuqua S.A.W., Brown R.W., Chamness G.C., Osborne C.K. & McGuire W.L. 1993. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J. Natl. Cancer Inst. 85(3):200-206. <http://dx.doi.org/10.1093/jnci/85.3.200>

Anborgh P.H., Mutrie J.C., Tuck A.B. & Chambers A.F. 2011. Pre- and post-translational regulation of osteopontin in cancer. J. Cell Commun. Signal. 5(2):111-122. <http://dx.doi.org/10.1007/s12079-011-0130-6> <PMid:21516514>

Andrade R.L.F.S., Oliveira D.M., Dantas A.F.M., Souza A.P., Neto P.I.N.R. & Riet-Correa F. 2012. Tumores de cães e gatos diagnosticados no semiárido da Paraíba. Pesq. Vet. Bras. 32(10):1037-1040. <http://dx.doi.org/10.1590/S0100-736X2012001000016>

Arias J.I., Paredes E. & Torres C.G. 2015. Mammary carcinoma in a male dog: clinical and immunohistochemical characterization. Arch. Med. Vet. 47:111-115.

Battisti M.K.B., Silva D.M., Reusing M.S.O., Beltrame O.C., Schmidt S.E.M., Fagliari J.J., Dittrich R.L. & Guérios S.D. 2013. Acute phase proteins in female dogs with mammary tumors. Ciência Rural 43(5):902-907. <http://dx.doi.org/10.1590/S0103-84782013000500025>

Bellahcène A., Castronovo V., Ogbureke K.U.E., Fisher L.W. & Fedarko N.S. 2008. Small Integrin-Binding LIgand N-linked Glycoproteins (SIBLINGs): multifunctional proteins in cancer. Nat. Rev. Cancer 8(3):212-226. <http://dx.doi.org/10.1038/nrc2345> <PMid:18292776>

Biermann K., Go¨ke F., Nettersheim D., Eckert D., Zhou H., Kahl P., Gashaw I., Schorle H. & Büttner R. 2007. c-KIT is frequently mutated in bilateral germ cell tumours and down-regulated during progression from intratubular germ cell neoplasia to seminoma. J. Pathol. 213(3):311-318. <http://dx.doi.org/10.1002/path.2225> <PMid:17768701>

Bongiovanni L., Romanucci M., Malatesta D., D'Andrea A., Ciccarelli A. & Della Salda L. 2015. Survivin and related proteins in canine mammary tumors: immunohistochemical expression. Vet. Pathol. 52(2):269-275. <http://dx.doi.org/10.1177/0300985814529312> <PMid:24686389>

Bramwell V.H., Tuck A.B., Chapman J.A., Anborgh P.H., Postenka C.O., Al-Katib W., Shepherd L.E., Han L., Wilson C.F., Pritchard K.I., Pollak M.N. & Chambers A.F. 2014. Assessment of osteopontin in early breast cancer: correlative study in a randomized clinical trial. Breast Cancer Res. 16(1):R8. <http://dx.doi.org/10.1186/bcr3600> <PMid:24451146>

Brodey R.S., Goldschmidt M.H. & Roszel J.R. 1983. Canine mammary gland neoplasms. J. Am. Anim. Hosp. Assoc. 19:61-90.

Bronson R.T. 1982. Variation in age at death of dogs of different sexes and breeds. Am. J. Vet. Res. 43(11):2057-2059.

Brown L.F., Papadopoulos-Sergiou A., Berse B., Manseau E.J., Tognazzi K., Perruzzi C.A., Dvorak H.F. & Senger D.R. 1994. Osteopontin expression and distribution in human carcinomas. Am. J. Pathol. 145(3):610-623. <PMid:8080043>

Burrai G.P., Tanca A., De Miglio M.R., Abbondio M., Pisanu S., Polinas M., Pirino S., Mohammed S.I., Uzzau S., Addis M.F. & Antuofermo E. 2015. Investigation of HER2 expression in canine mammary tumors by antibody-based, transcriptomic and mass spectrometry analysis: is the dog a suitable animal model for human breast cancer? Tumour Biol. 36(11):9083-9091. <http://dx.doi.org/10.1007/s13277-015-3661-2> <PMid:26088453>

Carecchio M. & Comi C. 2011. The role of osteopontin in neurodegenerative diseases. J. Alzheimers Dis. 25(2):179-185. <http://dx.doi.org/10.3233/JAD-2011-102151> <PMid:21358042>

Carvalho M.I., Guimarães M.J., Pires I., Prada J., Silva-Carvalho R., Lopes C. & Queiroga F.L. 2013. EGFR and microvessel density in canine malignant mammary tumors. Res. Vet. Sci. 95(3):1094-1099. <http://dx.doi.org/10.1016/j.rvsc.2013.09.003> <PMid:24091029>

Chambers A.F., Wilson S.M., Kerkvliet N., O'Malley F.P., Harris J.F. & Casson A.G. 1996. Osteopontin expression in lung cancer. Lung Cancer 15(3):311-323. <http://dx.doi.org/10.1016/0169-5002(95)00595-1> <PMid:8959677>

Chang P.L., Harkins L., Hsieh Y.H., Hicks P., Sappayatosok K., Yodsanga S., Swasdison S., Chambers A.F., Elmets C.A. & Ho K.J. 2008. Osteopontin expression in normal skin and non-melanoma skin tumors. J. Histochem. Cytochem. 56(1):57-66. <http://dx.doi.org/10.1369/jhc.7A7325.2007> <PMid:17938278>

Coppola D., Szabo M., Boulware D., Muraca P., Alsarrai M., Chambers A.F. & Yeatman T.J. 2004. Correlation of osteopontin protein expression and pathological stage across a wide variety of tumor histologies. Clin. Cancer Res. 10(Pt 1):184-190. <http://dx.doi.org/10.1158/1078-0432.ccr-1405-2> <PMid:14734468>

Daleck C.R., De Nardi A.B. & Rodaski S. 2016. Oncology in Dogs and Cats. 2nd ed. Roca, Rio de Janeiro, p.2-7.

Damasceno K.A., Ferreira E., Estrela-Lima A., Bosco Y., Silva L.P., Barros A.L.B., Bertagnolli A.C. & Cassali G.D. 2016a. Relationship between the expression of versican and EGFR, HER-2, HER-3 and CD44 in matrix-producing tumors in the canine mammary gland. Histol. Histopathol. 31(6):675-688. <http://dx.doi.org/10.14670/HH-11-705>

Damasceno K.A., Ferreira E., Estrela-Lima A., Gamba C.O., Miranda F.F., Alves M.R., Rocha R.M., de Barros A.L. & Cassali G.D. 2016b. HER-2 and EGFR mRNA expression and its relationship with versican in malignant matrix-producing tumors of the canine mammary gland. PLoS One 11(8):e0160419. <http://dx.doi.org/10.1371/journal.pone.0160419> <PMid:27490467>

De las Mulas M.J., Ordás J., Millan Y., Fernandez-Soria V. & Ramon y Cajal S. 2003. Oncogene HER-2 in canine mammary gland carcinomas: an immunohistochemical and chromogenic in situ hybridization study. Breast Cancer Res. Treat. 80(3):363-367. <http://dx.doi.org/10.1023/a:1024929730165> <PMid:14503809>

De Nardi A.B., Daleck C.R., Amorin R.L., Huppes R.R., Uscategui R.A.R., Rodaski S., Calderon C. & Neto R.T. 2013. Cyclooxigenase-2 expression in primary metastatic and nonmetastatic canine mammarian carcinomas. Arch. Med. Vet. 45:311-316. <http://dx.doi.org/10.4067/S0301-732X2013000300012>

Denhardt D.T., Mistretta D., Chambers A.F., Krishna S., Porter J.F., Raghuram S. & Rittling S.R. 2003. Transcriptional regulation of osteopontin and the metastatic phenotype: evidence for a Ras-activated enhancer in the human OPN promoter. Clin. Exp. Metastasis 20(1):77-84. <http://dx.doi.org/10.1023/a:1022550721404> <PMid:12650610>

Dias M.L.M., Andrade J.M.L., Castro M.B. & Galera P.D. 2016. Survival analysis of female dogs with mammary tumors after mastectomy: epidemiological, clinical and morphological aspects. Pesq. Vet. Bras. 36(3):181-186. <http://dx.doi.org/10.1590/S0100-736X2016000300006>

Dobson J.M. 2013. Breed-Predispositions to cancer in pedigree dogs. ISRN Vet Sci. 2013:941275. <http://dx.doi.org/10.1155/2013/941275> <PMid:23738139>

Eccles S.A., Modjtahedi H., Box G., Court W., Sandle J. & Dean C.J. 1995. Significance of the c-erbB family of receptor tyrosine kinases in metastatic cancer and their potential as targets for immunotherapy. Invasion Metastasis 14(1/6):337-348. <PMid:7657527>

Elebro J., Heby M., Warfvinge C.F., Nodin B., Eberhard J. & Jirström K. 2016. Expression and prognostic significance of human epidermal growth factor receptors 1, 2 and 3 in periampullary adenocarcinoma. Plos One 11(4):e0153533. <http://dx.doi.org/10.1371/journal.pone.0153533> <PMid:27070783>

Feliciano M.A.R., Silva A.S., Peixoto R.V.R., Galera P.D. & Vicente W.R.R. 2012. Estudo clínico, histopatológico e imunoistoquímico de neoplasias mamárias em cadelas. Arq. Bras. Med. Vet. Zootec. 64:1094-1100. <http://dx.doi.org/10.1590/S0102-09352012000500002>

Ferreira E., Bertagnoli A.C., Gobbi H. & Canali G.D. 2014. HER-2 gene expression in atypical ductal hyperplasia associated with canine mammary carcinomas. Arq. Bras. Med. Vet. Zootec. 66(2). <http://dx.doi.org/10.1590/1678-41626212>

Fighera R.A., Souza T.M., Silva M.G., Brum J.S., Graça D.L., Kommers G.D., Irigoyen L.F. & Barros C.S.L. 2008. Causas de morte e razões para eutanásia de cães da Mesorregião do Centro Ocidental Rio-Grandense (1965-2004). Pesq. Vet. Bras. 28(4):223-230. <http://dx.doi.org/10.1590/S0100-736X2008000400005>

Fisher L.W., Torchia D.A., Fohr B., Young M.F. & Fedarko N.S. 2001. Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. Biochem. Biophys. Res. Commun. 280(2):460-465. <http://dx.doi.org/10.1006/bbrc.2000.4146> <PMid:11162539>

Frey A.B., Wali A., Pass H. & Lonardo F. 2007. Osteopontin is linked to p65 and MMP-9 expression in pulmonary adenocarcinoma but not in malignant pleural mesothelioma. Histopathology 50(6):720-726. <http://dx.doi.org/10.1111/j.1365-2559.2007.02675.x> <PMid:17493236>

Gama A., Gärtner F., Alves A. & Schmitt F. 2009. Immunohistochemical expression of Epidermal Growth Factor Receptor (EGFR) in canine mammary tissues. Res. Vet. Sci. 87(3):432-437. <http://dx.doi.org/10.1016/j.rvsc.2009.04.016> <PMid:19464036>

Graham J.C. & Myers R.K. 1999. The prognostic significance of angiogenesis in canine mammary tumors. J. Vet. Intern. Med. 13(5):416-418. <http://dx.doi.org/10.1892/0891-6640(1999)013<0416:tpsoai>2.3.co;2> <PMid:10499723>

Hirano Y., Aziz M., Yang W.L., Wang Z., Zhou M., Ochani M., Khader A. & Wang P. 2015. Neutralization of osteopontin attenuates neutrophil migration in sepsis-induced acute lung injury. Crit. Care 19:53. <http://dx.doi.org/10.1186/s13054-015-0782-3> <PMid:25887405>

Hirota S., Ito A., Nagoshi J., Takeda M., Kurata A., Takatsuka Y., Kurata A., Takatsuka Y., Kohri K., Nomura S. & Kitamura Y. 1995. Expression of bone matrix protein messenger ribonucleic acids in human breast cancers. Possible involvement of osteopontin in development of calcifying foci. Lab. Invest., J. Technical Methods Pathol. 72(1):64-69. <PMid:7837792>

Kahles F., Findeisen H.M. & Bruemmer D. 2014. Osteopontin: a novel regulator at the cross roads of inflammation, obesity and diabetes. Mol. Metab. 3(4):384-393. <http://dx.doi.org/10.1016/j.molmet.2014.03.004> <PMid:24944898>

Kamath S. & Buolamwini J.K. 2006. Targeting EGFR and HER-2 receptor tyrosine kinases for cancer drug discovery and development. Med. Res. Rev. 26(5):569-594. <http://dx.doi.org/10.1002/med.20070> <PMid:16788977>

Kandioler-Eckersberger D., Ludwig C., Rudas M., Kappel S., Janschek E., Wenzel C., Schlagbauer-Wadl H., Mittlböck M., Gnant M., Steger G. & Jakesz R. 2000. TP53 mutation and p53 overexpression for prediction of response to neoadjuvant treatment in breast cancer patients. Clin. Cancer Res. 6(1):50-56. <PMid:10656431>

Kim Y.W., Park Y-K. & Lee J. 1998. Expression of osteopontin and osteonectin in breast cancer. J. Korean Med. Sci. 13(6):652-657. <http://dx.doi.org/10.3346/jkms.1998.13.6.652> <PMid:9886175>

Klopfleisch R., Klose P. & Gruber A.D. 2010. The combined expression pattern of BMP2, LTBP4, and DERL1 discriminates malignant from benign canine mammary tumors. Vet. Pathol. 47(3):446-454. <http://dx.doi.org/10.1177/0300985810363904> <PMid:20375427>

Lee C.H., Kim W.H., Lim J.H., Kang M.S., Kim D.Y. & Kweon O.K. 2004. Mutation and overexpression of p53 as a prognostic factor in canine mammary tumors. J. Vet. Sci. 5(1):63-69. <PMid:15028887>

Lemmon M.A. & Schlessinger J. 2010. Cell signaling by receptor tyrosine kinases. Cell 141(7):1117-1134. <http://dx.doi.org/10.1016/j.cell.2010.06.011> <PMid:20602996>

Li H.T., Song X.Y., Yang C., Li Q., Tang D., Tian W.R. & Liu Y. 2013. Effect of hematoporphyrin monomethyl ether-mediated PDT on the mitochrondria of canine breast cancer cells. Photodiagnosis and photodynamic therapy. Photodiagnosis Photodyn. Ther. 10(4):414-421. <http://dx.doi.org/10.1016/10.1016/j.pdpdt.2013.03.005> <PMid:24284094>

Li Y., Jiang W., Wang H., Deng Z., Zang C., Tu M., Li L., Xiao W., Gao S., Luo W. & Lei G. 2016. Osteopontin promoters expression of matrix metalloproteinase 13 through NF-κB signaling in osteoarthirtis. Biomed. Res. Int. 2016:6345656. <http://dx.doi.org/10.1155/2016/6345656>

Liang J., Wu Y.L., Chen B.J., Zhang W., Tanaka Y. & Sugiyama H. 2013. The C-kit receptor-mediated signal transduction and tumor-related diseases. Int. J. Biol. Sci. 9(5):435-443. <http://dx.doi.org/10.7150/ijbs.6087> <PMid:23678293>

Liaw L., Birk D.E., Ballas C.B., Whitsitt J.S., Davidson J.M. & Hogan B.L. 1998. Altered wound healing in mice lacking a functional osteopontin gene (spp1). J. Clin. Invest. 101(7):1468-1478. <http://dx.doi.org/10.1172/JCI2131> <PMid:9525990>

Lin J., Myers A.L., Wang Z., Nancarrow D.J., Ferrer-Torres D., Handlogten A., Leverenz K., Bao J., Thomas D.G., Wang T.D., Orringer M.B., Reddy R.M., Chang A.C., Beer D.G. & Lin L. 2015. Osteopontin (OPN/SPP1) isoforms collectively enhance tumor cell invasion and dissemination in esophageal adenocarcinoma. Oncotarget 6(26):22239-22257. <http://dx.doi.org/10.18632/oncotarget.4161> <PMid:26068949>

Luo X., Ruhland M.K., Pazolli E., Lind A.C. & Stewart S.A. 2011. Osteopontin stimulates preneoplastic cellular proliferation through activation of the MAPK pathway. Mol. Cancer Res. 9(8):1018-1029. <http://dx.doi.org/10.1158/1541-7786.MCR-10-0472> <PMid:21673011>

Marmor M.D., Kochupurakkal B.S. & Yarden Y. 2004. Signal transduction and oncogenesis by ErbB/HER receptors. Int. J. Radiat. Oncol. Biol. Phys. 58(3):903-913. <http://dx.doi.org/10.1016/j.ijrobp.2003.06.002> <PMid:14967450>

Matsuyama S., Nakamura M., Yonezawa K., Shimada T., Ohashi F., Takamori Y. & Kubo K. 2001. Expression patterns of the erbB subfamily mRNA in canine benign and malignant mammary tumors. J. Vet. Med. Sci. 63:949-954. <http://dx.doi.org/10.1292/jvms.63.949> <PMid:11642281>

Michell A.R. 1999. Longevity of british breeds of dogs and its relationships with sex, size, cardiovascular variables and disease. Vet. Rec. 145(22):625-629. <http://dx.doi.org/10.1136/vr.145.22.625> <PMid:10619607>

Misdorp W., Else W. & Hellmen E. 1999. Histological classification of mammary tumors of the dog and cat. WHO International Histological Classification of Tumours of Domestic Animals. 2nd ed. Armed Forces Institute of Pathology, American Registry of Pathology, Washington DC.

Ng L., Wan T.M., Lam C.S., Chow A.K., Wong S.K., Man J.H., Li H.S., Cheng N.S., Pak R.C., Cheung A.H., Yau T.C., Lo O.S., Foo D.C., Poon J.T., Poon R.T., Pang R.W. & Law W.L. 2015. Post-operative plasma osteopontin predicts distant metastasis in human colorectal cancer. PLoS One 10(5):e0126219. <http://dx.doi.org/ 10.1371/journal.pone.0126219> <PMid:25961724>

Nieto A., Peña L., Pérez-Alenza M.D., Sánchez M.A., Flores J.M. & Castaño M. 2000. Immunohistologic detection of estrogen receptor alpha in canine mammary tumors: clinical and pathologic associations and prognostic significance. Vet. Pathol. 37(3):239-247. <http://dx.doi.org/10.1354/vp.37-3-239> <PMid:10810988>

Pawlowski K.M., Maciejewski H., Dolka I., Mol J.A., Motyl T. & Król M. 2013. Five markers useful for the distinction of canine mammary malignancy. BMC Vet. Res. 9:138. <http://dx.doi.org/10.1186/1746-6148-9-138> <PMid:23844591>

Peña L., Gama A., Goldschmidt M.H., Abadie J., Benazzi C., Castagnaro M., Díez L., Gärtner F., Hellmén E., Kiupel M., Millán Y., Miller M.A., Nguyen F., Poli A., Sarli G., Zappulli V. & de las Mulas J.M. 2014. Canine mammary tumors: a review and consensus of standard guidelines on epithelial and myoepithelial phenotype markers, HER2, and hormone receptor assessment using immunohistochemistry. Vet. Pathol. 51(1):127-145. <http://dx.doi.org/10.1177/0300985813509388> <PMid:24227007>

Proschowsky H.F., Rugbjerg H., & Ersboll A.K. 2003. Mortality of purebred and mixed-breed dogs in Denmark. Prev. Vet. Med. 58(1/2):63-74. <http://dx.doi.org/10.1016/s0167-5877(03)00010-2> <PMid:12628771>

Psyrri A., Kalogeras K.T., Wirtz R.M., Kouvatseas G., Karayannopoulou G., Goussia A., Zagouri F., Veltrup E., Timotheadou E., Gogas H., Koutras A., Lazaridis G., Christodoulou C., Pentheroudakis G., Economopoulou P., Laskarakis A., Arapantoni-Dadioti P., Batistatou A., Sotiropoulou M., Aravantinos G., Papakostas P., Kosmidis P., Pectasides D. & Fountzilas G. 2017. Association of osteopontin with specific prognostic factors and survival in adjuvant breast cancer trials of Hellenic Cooperative Oncology Group. J. Transl. Med. 15(1):30. <http://dx.doi.org/10.1186/s12967-017-1134-7> <PMid:28193231>

Rangaswami H., Bulbule A., Kundu G.C. 2006. Osteopontin: role in cell signaling and cancer progression. Trends Cell Biol. 16(2):79-87. <http://dx.doi.org/10.1016/j.tcb.2005.12.005> <PMid:16406521>

Rittling S.R. & Novick K.E. 1997. Osteopontin expression in mammary gland development and tumorigenesis. Cell Growth Differ. 8(10):1061-1069. <PMid:9342184>

Rittling S.R. & Singh R. 2015. Osteopontin in immune-mediated diseases. J. Dent. Res. 94(12):1638-1645. <http://dx.doi.org/10.1177/0022034515605270> <PMid:26341976>

Rodrigues L.R., Lopes N., Sousa B., Vieira D., Milanezi F. & Paulsson M. 2009. Significance of osteopontin expression in human invasive breast tumor stroma. Open Breast Cancer J. 1:1-9. <http://dx.doi.org/10.2174/1876817200901010001>

Salas Y., Aburto E., Alonso R.A., Márquez A., Corona H. & Romero L. 2016. Association of histological features with potential risk factors and survival in canine mammary tumors. Vet. México OA 3(1). <http://dx.doi.org/10.21753/vmoa.3.1.359>

Salas Y., Márquez A., Diaz D. & Romero L. 2015. Epidemiological study of mammary tumors in female dogs diagnosed during the period 2002-2012: a growing animal health problem. PLoS One 10(5):e0127381. <http://dx.doi.org/10.1371/journal.pone.0127381> <PMid:25992997>

Santos I.F.C., Cardoso J.M.M., Oliveira K.C., Laisse C.J.M. & Bessa S.A.T. 2013. Prevalência de neoplasias diagnosticadas em cães no Hospital Veterinário da Universidade Eduardo Mondlane, Moçambique. Arq. Bras. Med. Vet. Zootec. 65(3):773-782. <http://dx.doi.org/10.1590/S0102-09352013000300025>

Santos M., Correia-Gomes C., Santos A., Matos A., Rocha E., Lopes C. & Pereira P.D. 2014. Nuclear pleomorphism: role in grading and prognosis of canine mammary carcinomas. Vet. J. 200(3):426-433. <http://dx.doi.org/10.1016/j.tvjl.2014.03.019> <PMid:24745769>

Sartin E.A., Barnes S., Kwapien R.P. & Wolfe L.G. 1992. Estrogen and progesterone receptor status of mammary carcinomas and correlation with clinical outcome in dogs. Am. J. Vet. Res. 53(11):2196-2200. <PMid:1466519>

Schnapper A. & Meyer W. 2004. Osteopontin distribution in the canine skeleton during growth and structural maturation. Cells Tissues Organs 178(3):158-167. <http://dx.doi.org/10.1159/000082246> <PMid:15655333>

Sebolt-Leopold J.S. & Herrera R. 2004. Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat. Rev. Cancer 4(12):937-947. <http://dx.doi.org/10.1038/nrc1503> <PMid:15573115>

Shevde L.A. & Samant R.S. 2014. Role of osteopontin in the pathophysiology of cancer. Matrix Biol. 37:131-141. <http://dx.doi.org/10.1016/j.matbio.2014.03.001> <PMid:24657887>

Silva I.L.D., Dias A.P.M., Bertagnolli A.C., Cassali G.P. & Ferreira E. 2014. Analysis of EGFR and HER-2 expressions in ductal carcinomas in situ in canine mammary glands. Arq. Bras. Vet. Zootec. 66(3):763-768. <http://dx.doi.org/10.1590/1678-41626128>

Singer J., Weichselbaumer M., Stockner T., Mechtcheriakova D., Sobanov Y,. Bajna E., Wrba F., Horvat R., Thalhammer J.G., Willmann M. & Jensen-Jarolim E. 2012. Comparative oncology: ErbB-1 and ErbB-2 homologues in canine cancer are susceptible to cetuximab and trastuzumab targeting. Mol. Immunol. 50(4):200-209. <http://dx.doi.org/10.1016/j.molimm.2012.01.002> <PMid:22424313>

Soler M., Domingues E., Lucas X., Novellas R., Gomes-Coelho K.V., Espada Y. & Agut A. 2016. Comparison between ultrasonographic findings of benign and malignant canine mammary gland tumors using B-mode, colour Doppler, power Doppler and spectral Doppler. Res. Vet. Sci. 107:141-146. <http://dx.doi.org/10.1016/j.rvsc.2016.05.015> <PMid:27473987>

Theocharis A.D., Skandalis S.S., Nell T., Murthaupt H.A.B., Hubo M., Frey H., Gopal S., Gomes A., Afratis N., Lim H.C., Couchman J.R., Filmus J., Sanderson R.D., Schaefer L., Iozzo R.V. & Karamanos N.K. 2015. Insights into the key roles of proteoglycans in breast cancer biology and translational medicine. Biochim. Biophys. Acta 1855(2):276-300. <http://dx.doi.org/10.1016/j.bbcan.2015.03.006> <PMid:25829250>

Tilli T.M., Bellahcène A., Castronovo V. & Gimba E.R. 2014. Changes in the transcriptional profile in response to overexpression of the osteopontin-c splice isoform in ovarian (OvCar-3) and prostate (PC-3) cancer cell lines. BMC Cancer 14:433. <http://dx.doi.org/10.1186/1471-2407-14-433> <PMid:24928374>

Trapp S.M., Iacuzio A.I., Barca Junior F.A., Kemper B., Silva L.C., Okano W., Tanaka N.M., Grecco F., da Cunha & Melo F. 2010. Causas de óbito e razões para eutanásia em uma população hospitalar de cães e gatos. Brazilian J. Vet. Res. Anim. Sci. 47(5):395-402. <https://doi.org/10.11606/issn.1678-4456.bjvras.2010.26821>

Tsai W.C., Lee H.S., Jin J.S., Gao H.W., Chao T.K., Chen A., Nieh S., Chan D.C., Nieh S., Chan D.C., Chang F.N. & Lin C.K. 2012. Association between osteopontin and EGFR expression with clinicopathological parameters in hepatocellular carcinoma. Chin. J. Physiol. 55(6):412-420. <http://dx.doi.org/10.4077/CJP.2012.BAA082> <PMid:23286449>

Tuck A.B, Hota C., Wilson S.M. & Chambers AF. 2003. Osteopontin-induced migration of human mammary epithelial cells involves activation of EGF receptor and multiple signal transduction pathways. Oncogene 22(8):1198-1205. <http://dx.doi.org/10.1038/sj.onc.1206209> <PMid:12606946>

Tuck A.B. & Chambers A.F. 2001. The role of osteopontin in breast cancer: clinical and experimental studies. J. Mammary Gland Biol. Neoplasia 6:419-429. <PMid:12013531>

Tuck A.B., O'Malley F.P., Singhal H., Harris J.F., Tonkin K.S., Kerkvliet N., Saad Z., Doig G.S. & Chambers A.F. 1998. Osteopontin expression in a group of lymph node negative breast cancer patients. Int. J. Cancer 79(5):502-508. <http://dx.doi.org/10.1002/(sici)1097-0215(19981023)79:5<502::aid-ijc10>3.0.co;2-3> <PMid:9761120>

Tuck A.B., O'Malley F.P., Singhal H., Tonkin K.S., Harris J.F., Bautista D. & Chambers A.F. 1997. Osteopontin and p53 expression are associated with tumor progression in a case of synchronous, bilateral, invasive mammary carcinomas. Arch. Pathol. Lab. Med. 121(6):578-584. <PMid:9199622>

Vollmann-Zwerenz A., Diermeier-Daucher S., Wege A.K., Sassen A., Schmidt-Brücken E. & Hofstaedter F. 2010. Multichromatic phenotyping of HER receptor coexpression in breast tumor tissue samples using flow cytometry-possibilities and limitations. Cytometry A 77(4):387-398. <http://dx.doi.org/10.1002/cyto.a.20868> <PMid:20151455>

Waller A.H., Sanchez-Ross M., Kaluski E. & Klapholz M. 2010. Osteopontin in cardiovascular disease: a potential therapeutic target. Cardiol. Rev. 18(3):125-131. <http://dx.doi.org/10.1097/CRD.0b013e3181cfb646> <PMid:20395697>

Weber G.F., Lett G.S. & Haubein N.C. 2010. Osteopontin is a marker for cancer aggressiveness and patient survival. Brit. J. Cancer 103(6):861-869. <http://dx.doi.org/10.1038/sj.bjc.6605834> <PMid:20823889>

Wei R., Wong J.P.C. & Kwok H.F. 2017. Osteopontin - a promising biomarker for cancer therapy. J. Cancer 8(12):2173-2183. <http://dx.doi.org/10.7150/jca.20480> <PMid:28819419>

West C.M., Joseph L. & Bhana S. 2008. Epidermal growth factor receptor targeted therapy. Brit. J. Radiol. 81:36-44. <http://dx.doi.org/10.1259/bjr/32798755>

Yamate T., Mocharla H., Taguchi Y., Igietseme J.U., Manolagas S.C. & Abe E. 1997. Osteopontin expression by osteoclast and osteoblast progenitors in the murine

bone marrow: demonstration of its requirement for osteoclastogenesis and its increase after ovariectomy. Endocrinology 138(7):3047-3055. <http://dx.doi.org/ 10.1210/endo.138.7.5285> <PMid:9202251>

Yan C.H., Lv M., Li H., Song X., Yan F., Cao S. & Ren X. 2015. Osteopontin is a novel prognostic biomarker in early-stage non-small cell lung cancer after surgical resection. J. Cancer Res. Clin. Oncol. 141(8):1371-1378. <http://dx.doi.org/10.1007/s00432-014-1902-1> <PMid:25555465>

Yarden Y. & Sliwkowski M.X. 2001. Untangling the erbB signaling network. Nat. Rev. Mol. Cell Biol. 2(2):127-137. <http://dx.doi.org/10.1038/35052073> <PMid:11252954>

Zacchetti A., Van Garderen E., Teske E., Nederbragt H., Dierendonck J.H. & Rutteman G.R. 2003. Validation of the use of proliferation markers in canine neoplastic and non-neoplastic tissues: comparison of KI-67 and proliferating cell nuclear antigen (PCNA) expression versus in vivo bromodeoxyuridine labelling by immunohistochemistry. APMIS 111(3):430-438. <10.1034/j.1600-0463.2003.t01-1-1110208.x> <PMid:12752223>

Zakaria R., Platt-Higgins A., Rathi N., Crooks D., Brodbelt A., Chavredakis E., Lawson D., Jenkinson M.D. & Rudland P. 2016. Metastasis-inducing proteins are widely expressed in human brain metastases and associated with intracranial progression and radiation response. Brit. J. Cancer 114(10):1101-1108. <http://dx.doi.org/ 10.1038/bjc.2016.103> <PMid:27100728>