Breast cancer and canine mammary cancer are malignancies that affect millions of bitches and women in the world. Several studies have shown a worse prognosis for patients with hypoxic mammary tumor (tumors with low oxygen perfusion) due to an increased expression of hypoxia-inducible factor (HIF) and consequently several other hypoxia-induced molecules, like vascular endothelial growth factor (VEGF), Lysyl-oxidase (LOX) and erythropoietin (EPO). All these molecules and factors contribute to a higher chance of tumor cells survival in this hypoxic microenvironment. In this paper review, molecular mechanisms which hypoxia influences on breast cancer were covered for both women and bitches, with some promising drugs that could target these molecules and increase patient survival and quality of life.

Key-words
Canine mammary tumor; hypoxia-inducible factor; HIF; VEGF; LOX.
INTRODUÇÃO

Breast cancer is a malignancy that affects the mammary gland. Malignant cells stop performing their role, and do not respect normal growth factor signaling. They are capable of multiply uncontrollably, without responding to any apoptosis signal. Although its occurrence is much higher in females of all species, males can also develop this type of cancer, which is considered very rare. In a retrospective study done in Mexico City and published in 2015, researchers evaluated 11,544 tissue biopsies collected from dogs during 11 years (2002-2012). From these biopsies, 1,917 (16.6%) corresponded to mammary gland lesions, with 47.8% being benign tumors and 47.5% malignant tumors (SALAS et al., 2015). In another study performed in a Brazilian city, from 126 neoplasia samples, 50 (39.7%) were mammary gland tumors, being only behind cutaneous tumor (46%) in occurrence (SOUZA et al. 2005). These studies show the importance of mammary gland tumors in small animal medicine.

Some tumors do not have the capacity to invade the tissue where it was originated. These tumors, called benign tumors, do not have the ability to metastasize (spread to other organs, colonize and multiply). In other cases, tumors acquire the ability to invade and metastasize to other organs, causing extensive damages. In these cases, tumors are called malignant or just cancer (WEINBERG, 2006).

Over the years, studies have shown a correlation between hypoxia and worst prognosis in many cancer types, including breast cancer (SCHINDL et al. 2002; BOS et al. 2003; DALES et al. 2005; GENERALI et al. 2006). In other words, many studies have demonstrated that there is a strong correlation between tumor cells under hypoxic conditions, and their invasiveness/metastases. Nowadays it is clear that although hypoxia can induce cell death by triggering apoptosis mechanisms, it can lead to the activation of adaptive mechanisms against hypoxia, leading to cell survival (ZHOU et al. 2006) and inducing breast cancer cells to be more aggressive, through regulation and expression of many genes (LIU et al. 2015). In humans, hypoxic breast cancer tumors can have an oxygen pressure as low as 10 mmHg, while the normal pressure of oxygen in the mammary gland is around 65 mmHg (VAUPEL et al. 1991).

Although the last paragraph was written based in human facts, we have good reasons to extrapolate these data for the canine mammary gland tumors. During years mice were used as a model for breast cancer in humans, but it was not a good model, because their tumors differed from women tumors, majorly in three aspects: laboratory rodents are highly inbred (genetically similar); their tumors contain viral particles; and they are pathologically different if compared to human tumors. Because of these facts, scientists saw that canine mammary tumors were good models to study human breast cancer, because they are similar both epidemiologically and pathologically (STRANDBERG and GOODMAN, 1974).

Considering the already known wide effect that hypoxia has on tumor cells, acting on the regulation on the expression of several genes, this literature review is going to focus in some mechanisms triggered by hypoxia that lead to a more invasive and aggressive breast cancer, in both women and bitches.

HIF-1 formation and degradation

Is it known that variation in oxygen levels causes physiological stimuli and leads to a response by all organisms. A very important molecule that plays a critical role in O2 sensing and response by cells is the hypoxia-inducible factor 1 (HIF-1), discovered in the 1990s by Wang and Semenza (WANG and SEMENZA, 1995).

HIF-1 is a complex molecule composed by two subunits, alpha (HIF-1α) and beta (HIF-1β), and these two units are capable of directly interacting with the DNA (WANG and SEMENZA, 1995), regulating the transcription of several genes (that are expressed under hypoxic conditions), including erythropoietin (EPO) and vascular endothelial growth factor (VEGF) genes (WANG and SEMENZA, 1993), two key genes in the production of new blood cells and new blood vessels, respectively.

It is important to note that HIF-1 is produced even in normoxic conditions. In these situations, an enzyme called proline hydroxylase uses the oxygen that is abundant in the cell to change the oxygen-dependent degradation domain (ODD), present in subunit alpha (HIF-1α), allowing pVHL (Von Hippel-Lindau protein) to recognize alpha subunit and bind to it, causing its ubiquination and consequent degradation by proteasomes. In this circumstance, without HIF-1α subunit, HIF-1 complex cannot be assembled, hence there is no action of HIF-1 on the regulation of several genes (BOS et al. 2003).

On the other hand, if the cell is exposed to hypoxia, the proline hydroxylase enzyme is inactivated (because of O2 low levels), there is no phosphorylation of alpha subunit, making possible for it to bind to beta subunit (HIF-1β), forming HIF-1 protein, that is going to bind to the DNA and coordinate the activation of over 1000 genes, generating multiple proteins (LIU et al. 2015).
HIF-1 role in tumor malignancy and invasiveness

Two researches done in 2002 and 2003 demonstrated a correlation between HIF-1α expression and prognosis in human patients with breast cancer. In one study, the scientists evaluated patients with positive lymph node breast cancer (metastatic). On the other hand, the second study used patients with negative lymph node breast cancer. In total, 359 patients were assessed, and levels of HIF-1α in the tumor cells were measured using immunohistochemical technique in tumor tissue embedded in paraffin (SCHINDL et al. 2002; BOS et al. 2003). In both studies scientists demonstrated that high levels of HIF-1α indicated a worst prognosis (correlated with survival time).

While normal cells rely on physiological angiogenesis, tumor cells can mimic it, creating their own blood supply. The angiogenic process in tumor cells involves many elements that are present in normal angiogenesis. As example of molecules involved in tumorigenic angiogenesis are: VEGF, fibroblast growth factor and interleukin-8. Among these molecules, VEGF is one of the most important factors in tumor angiogenesis and its expression and/or suppression of genes, resulting in the synthesis of different proteins. The process of metastasis is not different: it is regulated by several genes. Cells that upregulate some of these genes have higher chances to successfully complete all the steps of invasion and metastasis. It is worth noting that it has been demonstrated that many of these genes (that turn cells more susceptible to invade and metastasize) are upregulated by HIF-1, under hypoxic conditions, turning breast cancer more invasive and more metastatic (LIU et al. 2015).

Other crucial step for tumor settlement in a distant organ is the epithelial-mesenchymal transition (EMT), which is regulated by several genes. Cells that upregulate some of these genes have higher chances to successfully complete all the steps of invasion and metastasis. It is worth noting that it has been demonstrated that many of these genes (that turn cells more susceptible to invade and metastasize) are upregulated by HIF-1, under hypoxic conditions, turning breast cancer more invasive and more metastatic (LIU et al. 2015).

Several steps are required for tumor cells to metastasize

The process of metastasis is complex and comprehend multiple steps. The first step that allows cells to detach from the primary tumor and reach blood vessels, is the epithelial-mesenchymal transition (EMT), which has been proved to make tumor cells more prompt to invade, hence metastasize (BOYER and VALLE, 2000). With EMT, tumor cells can get into blood vessels, process that is called intravasation. Then, tumor cells need to resist to the blood vessels pressure and to the trip through different vessels sizes. Finally, these cells are going to be trapped in some organs, where they can adhere to the vessels walls, and extravasate to the surrounding tissue (FIDLER, 2003).

It is widely known that our cell machinery is regulated by the expression and/or suppression of genes, resulting in the synthesis of different proteins. The process of metastasis is not different: it is regulated by several genes. Cells that upregulate some of these genes have higher chances to successfully complete all the steps of invasion and metastasis. It is worth noting that it has been demonstrated that many of these genes (that turn cells more susceptible to invade and metastasize) are upregulated by HIF-1, under hypoxic conditions, turning breast cancer more invasive and more metastatic (LIU et al. 2015).

Other crucial step for tumor settlement in a distant organ is the modification of the metastatic niche. The metastatic niche is composed by the extracellular matrix (ECM), and stromal cells, that are going to support and feed the tumor cells. Other essential function of the metastatic niche is to provide anchorage to tumor cells, through adhesion molecules like β-catenin. Besides, there is production of factors that are important for the tumor cells survival and growth, e.g. VEGF, a very known molecule that causes angiogenesis (physiologically and tumorigenic) and has been described to initiate the pre-metastatic niche (LI and NEAVES, 2006).

VEGF is important for the preparation of metastatic niche

As already mentioned before, VEGF is one of the most important molecules secreted by tumors and stromal cells, related to tumor angiogenesis. VEGF is responsible for acting on Vascular Endothelial Growth Factor Receptors (VEGFR) - a family of receptors that are present in endothelium cells - which trigger a series of events when stimulated, leading to new blood vessels formation, among other functions. The most important family members are: VEGFR-1, VEGFR-2 and VEGFR-3, present in different tissues and expressing other functions. The most important family members are: VEGFR-1, VEGFR-2 and VEGFR-3, present in different tissues and expressing different functions (PAPETTI and HERMAN, 2002).

Studies done throughout the years have shown that a variety of Bone Marrow-Derived Cells (BMDCs) is important in facilitating malignant growth. Immunosuppressive cells, mesenchymal stem cells (MSCs), fibroblasts and macrophages are examples of cells that are included in the BMDCs family. These cells have crucial importance in providing tumor cells with mechanisms to evade the primary tumor site, “travel” to the metastasis location, and settle in the new tissue (KOH and KANG, 2012).
In a study done with mice, scientists looked for the importance of BMDCs VEGFR1+ (in other words, BMDCs that had VEGF receptor 1) in generating metastatic cluster, compared with BMDCs VEGFR1-. The results showed that BMDCs VEGFR1- did not produce pre-metastatic clusters. On the other hand, BMDCs VEGFR1+ formed pre-metastatic cluster, and many micrometastases with abnormal vasculature were found in the mice lungs. These results exemplify the importance of VEGFR1 and consequently importance of VEGF that is being overexpressed in tumor cells under hypoxia (KAPLAN et al. 2005).

**VEGF overexpression and breast cancer patients prognosis**

A review study looked at several papers published along the years, with almost 3000 human patients evaluated. In the end of the review, the author reaffirmed that breast cancer is dependent of angiogenic mechanisms, and VEGF plays a big role in the malignancy and progression of this type of cancer. Although some studies are controversial in determining VEGF as a good marker for patients prognosis, VEGF importance for breast cancer progression and metastasis has been proved (GASPARINI, 2000).

Although correlations between VEGF expression and cancer malignancy has been done in humans, in veterinary oncology, this field is just now being explored. In a study published in 2010, it was investigated the expression of VEGF in inflammatory canine mammary carcinoma (IMC) versus non-inflammatory mammary carcinomas. They concluded that VEGF expression in IMC was significantly higher when compared to the expression in non-IMC.

(MILLANTA et al. 2010). Another one evaluated the expression intensity of VEGF by canine mammary tumor cells, and results showed that malignant cells had stronger immunolabeling (when compared to benign tumor cells) and larger granules diffusely found in the cells cytoplasm (RESTUCCI et al. 2002).

Scientists from Japan were bolder and tried to determine the clinical significance of circulating VEGF (and not the VEGF found within the tumor) in bitches with tumors of the mammary gland. They found that animals with a mammary tumor had more circulating VEGF when compared to those without mammary tumors, and dogs with malignant mammary tumor had even higher concentration of circulating VEGF when compared to those with benign mammary tumors (KATO et al. 2007). Although studies associating VEGF concentrations to the patient prognosis (e.g. overall survival, response to treatment) were not found, the existing studies corroborate with the consolidation of VEGF measurement as a promising predictor factor to patient prognosis.

However, not all studies show a correlation between higher VEGF expression with increased malignancy. In a 2006 study, a correlation between VEGF increased expression and a poorer prognostic in canine patients was not established (MILLANTA et al. 2006). It was shown the necessity of more studies in this very new field in veterinary medicine.

**Lysyl oxidase (LOX): importance in physiological and cancerous situations**

LOX is an enzyme part of the LOX family that includes 5 molecules (LOX and LOX-like 1 to 4). This enzyme family is known for being copper-dependent and fundamental for ECM formation and maintenance. It has been demonstrated its importance in cancer development and in physiological events (GRAU-BOVÉ et al. 2015).

One of the main importance of LOX is the polymerization of different collagen fibers in the ECM. Collagen fibers are essential for normal tissues and tumor tissues, because it supports cells (tensile strength), and it is important in cell adhesion, chemotaxis and migration (very important in inflammatory responses) and tissue development (FRANTZ et al. 2010). The polymerization of these fibers occurs by the oxidation of another enzyme, the peptidyl lysine, that convert lysine residues into α-amino adipic- δ-semialdehyde. In this process, elastin and collagen chains are stabilized, making the ECM resistant enough to support cells (LUCERO and KAGAN, 2006). It was demonstrated LOX importance in skin, cardiac and lung cells. In one experiment, scientists deactivated the expression of the LOX gene in mice and these animals had cardiovascular malformation, reduced elasticity of skin and lungs, problems in their airway formation, among other alterations (MÄKI et al. 2005).

On the other hand, in tumor cells, an increase in HIF-1, leaded by hypoxia, can result in LOX overexpression that is going to act on the ECM directly on the polymerization of collagen fibers (MAKRIS et al. 2014), allowing breast tumor cells to invade and metastasize (ERLER et al. 2006). Other studies corroborate to the importance of LOX in tumor cells metastases: highly metastatic human breast cancer cells had higher LOX expression in comparison to poorly metastatic cells (KIRSCHMANN et al. 2002); cells with high invasion rate had their invasion impaired when LOX expression was blocked with a drug (PAYNE et al. 2005).

Animal researches about the importance of LOX in tumor development and angiogenesis are hardly found. One study looked for genes that are overregulated or down regulated in canine mammary tumors. A gene called Lysyl oxidase-like 2, related to the LOX gen family, was found to be overexpressed in cases of mammary tumors (RAO et al. 2009). Another study used many past experiments in animals using a copper-antagonize drug, and showed some effect in the cancer treatment, due to the drug capability of preventing angiogenesis, hence tumor progression.
LOX expression and tumor aggressiveness

Many studies have shown a positive correlation between LOX expression and higher cell motility and invasiveness and increased aggression in tumors. A study published in 2005 compared concentrations of LOX in different human breast cancer tumors with different malignancy. It was found that more aggressive breast cancers exhibited much higher concentration of LOX than less aggressive breast cancer or normal breast tissue (with normal expression of LOX) (PAYNE et al. 2005). A correlation between LOX expression and cancer patients prognosis has also been studied, not only for breast cancer, but also for other different types of cancer as the oropharyngeal squamous cell carcinoma (OSCC), a family member of the head and neck squamous cell carcinoma (HNSCC), that is a very aggressive type of cancer. One of these studies focusing on OSCC tried to describe a correlation between LOX expression and patients overall survival. 267 patients were analysed, and the results showed that an increased LOX expression was significantly correlated with lower overall survival, not only for OSCC patients, but for patients with oral cavity OSCC as well (SHIEH et al. 2011). Another study resulted in similar data found by Shieh et al. (2011), where increased expression of LOX was correlated with more malignant and lower patient overall survival (ALBINGER-HEGYI et al. 2009).

Friesenengst (2014) and collaborators correlated LOX expression and breast cancer patient prognosis. The results showed that patients with estrogen-receptor (ER) negative breast cancer with higher expression of LOX had a worst prognosis, with lower disease free survival and metastasis free survival, when compared with patients with low LOX expression. However, overexpression of LOX seemed not to affect the overall survival of ER-negative or ER-positive breast cancer patients. Although there are some controversies, several studies demonstrate higher LOX expression being related with patient poorer prognosis in different types of cancer. Unfortunately, no studies establishing the importance of LOX expression and tumor progression in animals were found.

HIF-1 overexpression and breast cancer prognosis

It was demonstrated in this review that HIF-1 is one of the major regulators of cellular response to hypoxia, coordinating the expression of several genes, leading to the production of different proteins with different functions. Now, looking in a broader way, some studies that show the relation between HIF-1 expression and breast cancer prognosis are going to be covered.

A study published in 2002 looked at the correlation between HIF-1α expression and prognosis of breast cancer lymph node positive patients. They analysed 260 patients that were treated by lumpectomy or mastectomy combined with adjuvant chemotherapy. The results showed that the 5 years overall survival was equal to 75.31% in patients with low HIF-1α expression, 61.26% in patients that had a moderate expression and, in patients with high HIF-1α expression, the percentage of 5-years overall survival was equal to 59.25% (SCHINDL et al. 2002).

Another study published just one year later, looked at the overexpression of HIF-1α in patients with lymph node negative breast cancer. The sample group used for this study was composed by 150 patients with breast carcinoma in early stage. They analysed overall survival, disease free survival, tumor size and lymph node status. For the patients with low expression of HIF-1α, 79% had a disease-free survival, against 67% of those patients with high expression of HIF-1α. About the overall survival, 89% of patients with low expression of HIF-1α survived, against 76% of the patients that composed the other group. This data shows a strong correlation between HIF-1α high expression and poorer prognosis (BOS et al. 2003).

Generali et al. (2006) point out the correlation between HIF-1α expression and the response to primary chemoendocrine therapy and disease-free survival in breast cancer patients. 187 breast cancer patients were analysed, and the results showed that patients with higher concentrations of HIF-1α in their tumors had a decreased response to chemotherapy. The authors also established a correlation between higher HIF-1α expression and lower disease-free survival.

Another research performed in humans with 745 breast cancer patients aimed to set up a correlation between HIF-1α expression and early relapse of cancer. HIF-1α increased expression was related to high metastasis risk, poor overall survival and higher chances of early relapse (DALES et al. 2005), as already observed in the previous studies presented in this review.

In the veterinary medicine, only now this study field is being explored. Until the publication of this review, papers establishing the correlation between high HIF-1 expression and its clinical relevance for the mammary tumor outcome were not found. However, there are other associations that can be made. In one study published in 2005, researchers were looking for the expression of erythropoietin receptors (EPOR) in canine mammary tumors. According to this paper, in a normal mammary gland, EPOR were almost never detected. The contrary happened in pre-neoplastic or neoplastic lesions: these receptors were always...
detected, and its concentration increased proportionally to the severity of the lesion (SFAC T E R I A et al. 2005). It is widely known that the erythropoietin (EPO) gene is more activated when the animal is under hypoxic situations. For example, an athlete in high altitudes is going to have a higher expression of the EPO gene, with higher production of red blood cells (RBC) consequently. In this process of hypoxia-induced EPO expression, HIF molecule has a crucial role. It is necessary for the EPO gene regulation, together with all the other genes that are overexpressed in hypoxic situations (STOCKMANN and FANDREY, 2006).

Another paper leads us to think about the importance of HIF-1 in canine mammary tumors. As the one described in the last paragraph, HIF-1 concentration and significance were not directly assessed. The molecule used as parameter was the epidermal growth factor receptor (EGFR), a receptor that is correlated to bad prognosis in human breast cancer. This receptor is thought to be overexpressed in hypoxic situations, where HIF production is high. Accordingly to CARVALHO et al. (2013), high levels of EGFR was significantly correlated to tumor size, necrosis, mitotic and histological grade, malignancy and clinical stage.

If we look at the animal researches, there is a good indication that, similarly to humans, HIF expression is associated with a worst outcome, because it is linked to the overexpression of genes that favor tumor malignancy, e.g. EPO and EGFR.

**HIF-1 as a therapy target**

For many years it has been demonstrated that HIF family is the maestro of cell response to hypoxia. It has been demonstrated that HIF-1 plays an important role in cancer cells aggressiveness and malignancy as well. Knowing the importance of HIF-1 for tumor development under hypoxic conditions, it became a desirable target for cancer therapy. Targeting the sub products of HIF-1 has been an option as well, achieving a more specific therapy with less side effects. These targets include VEGF and LOX, which functions and importance have been described in this review.

A variety of drugs has been tested or is already approved for the blockade of HIF-1, in different phases of its formation. Some drugs inhibit the first steps for the generation of HIF-1. For example, the EZN-2968 drug (still in clinical trial), is a third-generation nucleotide that inhibits the formation and expression of HIF-1α mRNA. In an experiment published in 2008, researchers evaluated the in vivo and in vitro capacity of this drug to act on tumor cells. In this study, the drug was used in human prostate carcinomas and glioblastoma cells, with a potent reduction in HIF-1α expression, in both normoxic and hypoxic environment. The experiments in vivo (using mice) revealed an intense downregulation of HIF-1α expression as well as VEGF, in mice liver (GREENBERGER et al. 2008). Other study with even more practical results, was a clinical trial, done in 2013. Although the trial has not been concluded, due to a cut on sponsorship, the preliminary results are exciting. Ten human patients with refractory advanced solid tumors were treated with EZN-2968, in a 2 hours intravenous infusion, once a week, during three weeks. A patient with duodenal neuroendocrine tumor had its disease progression stabilized for 24 weeks and HIF-1α mRNA levels were reduced in 4 of 6 patients (JEONG et al. 2014). Although this study was interrupted, data presented is promising and deserve more study in this area.

If we look at the drugs that have an impact over HIF-1 production and are already approved by the United States Food and Drug Administration (FDA), we have some examples like the Bortezomib. In some cases, drugs that have been used for years just now are being recognized by its capacity of blocking or impairing the production of molecules that are expressed under hypoxia.

Bortezomib is a proteasome inhibitor approved by FDA for the treatment of multiple myeloma that did not respond to the usual treatment. In more recent studies, it has been elucidated Bortezomib action on the suppression of HIF-1 formation. This action is believed to be due to an inhibition in the recruitment of a HIF-1α co-activator, called p300, and other impairments in the molecular path for HIF-1 formation (B E F A N I et al. 2012; SHIN et al. 2008). In Brazil, this drug has been approved by the National Health Surveillance Agency (ANVISA) since 2005 for the treatment of multiple myeloma, but its use is not covered by the National Healthcare System (SUS) (MINISTÉRIO DA SAÚDE, 2010). Veterinary Medicine studies evaluated the treatment of canine patients with Bortezomib and found satisfactory results for the treatment of canine malignant melanomas and lymphomas (ITO et al. 2013; KOJIMA et al. 2013). Precursor studies were developed evaluating the action of clinical usage of Bortezomib in human breast cancer patients, with not too many exciting results (CODONY-SERVAT et al. 2006; YANG et al. 2006).

Other drugs are more specific for the blockage of the molecules which production is stimulated by HIF-1 factor. For example, among many drugs that target VEGF, one of the most used is the drug called bevacizumab (Avastin®). It was approved by the FDA for the treatment of glioblastoma (used alone) or in combination with regular chemotherapy for the treatment of metastatic colorectal cancer, non-small cell lung cancers and metastatic renal cell cancer (NATIONAL CANCER INSTITUTE, 2015). This drug is actually a monoclonal antibody, produced by mice, that is able to ligate and inactivate human VEGF (FERRARA et al. 2004). In an animal clinical trial, nine dogs with soft tissue sarcoma were immunized with a VEGF human vaccine. All the dogs developed antibodies against both human and canine VEGF, and the tumor response...
was seen in 30% of the dogs, concluding a possible use of this immunotherapy in canine patients (KAMSTOCK et al. 2007).

Many drugs have been tested for the inhibition of LOX. It seems that there is no approved drug by the FDA for the inhibition of LOX in breast cancer patients, although there are some studies showing the inhibition power of drugs like homocysteine thiolactone and beta-aminopropionitrile (BONDAVERA et al. 2009; LIU et al. 1997).

**Conclusion**

This review shows the broad effect, almost always negative, that hypoxia has on both human and animal mammary tumor cells. In many cases hypoxia can turn tumor cells more malignant not only because of VEGF or LOX upregulation, but also by a lot of other mechanisms. HIF-1 is one of the major regulators, and its blockage or the inhibition of its products would be valuable for cancer therapy. Although there are some drugs in the market, more studies are necessary for both better understanding the mechanisms of aggressiveness driven by hypoxia and discovering better drug options for the treatment of hypoxic breast cancers. We should use these new molecular tools in order to do a more accurate tumor staging, and to prescribe a more personalized and directed treatment for each patient. Hypoxic factors and targeting them are new subjects in both human and animal medicine, so we should take advantage on this and work together, to find better ways to treat our patients, giving them what they need to get better or at least to have quality of life.
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