Universidade Trás-os-Montes e Alto Douro

Immunohistochemical expression of αB-crystallin and L1CAM in canine mammary tumors

Masters Dissertation in Veterinary Medicine

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SIDE NOTE

This masters dissertation was elaborated as an integrant part of my internship of the degree in Veterinary Medicine.

My internship consisted in a 3 month stay in Madrid, accompanying the work of Prof. Dr. Laura Peña in the Veterinary Pathology Laboratory of the Department of Animal Medicine, Surgery and Pathology at the Complutense University of Madrid. During this stay, besides our research in L1 CAM, I participated in weekly joint sessions for the exam of the European College of Veterinary Pathology, as well as monthly journal sessions. I also did necropsies, biopsy diagnosis and helped around the normal routine of the laboratory.

I also had a small stay in Vila Real, at the Trás-os-Montes e Alto Douro University, of less more than 1 month, were I accompanied the work of Prof. Dr. Adelina Gama Quaresma, where we did our research with CRYAB.

Besides the work of this dissertation, I also did a 4-month internship in the Baixo-Vouga Veterinary Hospital, where I got the chance to develop my medical skills. At this time I was also able to be involved in the normal routine of the laboratory of the hospital, where blood and urine samples and cytologies were available to evaluation every day, so I also developed my clinical pathology skills.

ABSTRACT

Human breast cancer is one of the most common neoplasms found in women, being the female dog the most accurate animal model to study it. Research on this field has been growing largely for the past years.

αB-Crystallin (CRYAB) is a member of the small heat shock proteins superfamily, implicated in cellular homeostasis and anti-apoptotic features, while L1 cell adhesion molecule (L1CAM) is a surface glycoprotein of the Ig's superfamily interacting with the extracellular matrix and other cells and more recently implicated in angiogenesis. In the present work, two separate immunohistochemical studies were performed in canine mammary tumors, one evaluating CRYAB and the other L1CAM expression.

To evaluate CRYAB expression in canine mammary tumors (CMT's) we used a series of 79 samples, composed by normal/hyperplastic tissues (n=9), benign tumors (n=15) and malignant tumors (n=55). Immunohistochemical evaluation was based on a semiquantitative analysis, according to the immunolabeling percentage (PS) and staining intensity (SI). A final score was obtained, based on the product PSxSI, with tissues classified as low (PSxSI<4) or high scores (PSxSI≥4). The goal was to evaluate the immunolabeling characteristics and to accomplish if there was any relation of CRYAB expression and clinicopathological characteristics, such as lymph-node metastasis or histological groups. Results showed a low expression of CRYAB by the myoepithelium of normal/hyperplastic tissues and a tendency to a higher expression in tumors (either benign or malignant), although not on a significant level. We found significant differences between histological groups regarding CRYAB expression (p=0.022). Positive expression was associated with the absence of lymph-node metastasis (p=0.014). CRYAB was mainly expressed by the myoepithelial cells of tumor samples, although not exclusively. Differences between histological groups suggest CRYAB may be a future factor of prognosis and probably an interesting therapeutic target. Further studies with a larger series are required to explain the differences obtained.

L1CAM expression was evaluated on 27 malignant, grade 3 CMT's, including inflammatory carcinomas (n=12) and non-inflammatory carcinomas (n=15). Immunohistochemical evaluation was based on a quantitative analysis, according to the immunolabeling percentage (PS) and the staining intensity (SI). A final score was obtained, rising from 0 or 2 to 8, by adding PS+SI. The goal was to evaluate the differences between inflammatory and non-inflammatory carcinomas. We also evaluated L1CAM expression by endothelial cells. Results showed significant differences between the inflammatory and the non-inflammatory group (p=0.003), with an overexpression

within the inflammatory group. The staining pattern was also significantly different (p=0.036). Regarding the endothelial cells, a positive correlation was found between the total score of L1CAM and the positivity of tumor and embolized vessels, but not on a significant level (p=0.05). Endothelial cells of normal vasculature showed an overexpression when compared to tumor ones. The latter showed 56% negativity in L1CAM expression (n=14). These results reveal, for the first time in CMT's, that L1CAM may be a potential therapeutic target in inflammatory carcinomas in female dogs, giving its differences between the two groups. Further studies should be performed in larger series to accomplish more information regarding this marker in the canine species.

Key Words: Canine mammary tumors; αB-Crystallin; L1CAM; cancer; breast

RESUMO

Os tumores de mama representam um dos tipos de cancro mais comuns na mulher, sendo que a cadela é apontada, atualmente, como o melhor modelo animal para o seu estudo. Assim sendo, a investigação neste ramo tem crescido a um ritmo elevado.

A αB-cristalina (CRYAB) pertence à superfamília das *small heat shock proteins,* influenciando a homeostasia celular e tendo funções anti-apoptóticas, enquanto a molécula de adesão celular L1 (L1CAM) é uma glicoproteína de superfície da família das imunoglobulinas, interagindo com a matriz extracelular e com outras células. Recentemente foi descrito o seu envolvimento na angiogénese. Neste trabalho, foram realizados dois estudos imunohistoquímicos independentes em tumores mamários caninos, tendo um avaliado a expressão do CRYAB e o outro a expressão do L1CAM.

Para avaliar a imunoexpressão do CRYAB em tumores mamários caninos, foram utilizadas 79 amostras, constituidas por tecidos normais/hiperplásicos (n=9), tumores benignos (n=15) e malignos (n=55). A avaliação foi feita com base num método semi-quantitativo, multiplicando a percentagem (PS) pela intensidade da imunomarcação (SI), obtendo o *score* total (PSxSI), posteriormente dividido em *score* alto (TS≥4) e baixo (TS<4). Os resultados revelaram uma baixa expressão do CRYAB ao nível do mioepitélio das amostras normais/ hiperplásicas e uma maior expressão em amostras tumorais. Encontrámos diferenças estatisticamente significativas relativamente à expressão do CRYAB entre diferentes grupos histológicos (p=0.022) e entre os diferentes tipos histológicos de neoplasias malignas, sendo mais frequentemente expresso em neoplasias com proliferação mioepitelial. A expressão do CRYAB encontrava-se também associada à ausência de metástases nos linfonodos (p=0.014). Consideramos necessários estudos com uma amostragem maior para confirmar as diferenças obtidas.

A expressão do L1CAM foi avaliada em 27 carcinomas mamários de alto grau histológico, incluindo 12 carcinomas inflamatórios e 15 não inflamatórios. A avaliação foi efetuada baseando-se na percentagem e intensidade de células coradas (PS e SI, respetivamente). O *score* final foi obtido somando PS+SI, obtendo valores 0 ou 2 a 8. O objetivo foi avaliar se existiam diferenças na expressão do L1CAM entre carcinomas inflamatórios e não inflamatórios. Os resultados revelaram diferenças entre o grupo inflamatório e o não inflamatório (p=0.003), com uma sobrexpressão no grupo inflamatório. O padrão de coloração apresentado pelas células também se revelou significativamente diferente (p=0.036). Relativamente às células endoteliais, encontrámos uma correlação positiva entre a expressão do L1CAM e a positividade de células endoteliais do tumor e do endotélio com êmbolos, mas não a níveis significativos (p=0.05).

Na vasculatura normal, observou-se sobrexpressão do L1CAM relativamente às células endoteliais do tumor, com 56% (n=14) das mesmas com expressão negativa. Estes resultados revelam, pela primeira vez em tumores mamários caninos, que o L1CAM poderá representar um alvo terapêutico em carcinoma inflamatório, dadas as diferenças encontradas entre os dois grupos. São necessários estudos posteriores, em séries mais numerosas, para reunir mais informação acerca desta molécula.

Palavras-chave: Tumores mamários caninos; αB-Cristalina; L1CAM; Cancro; Mama

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ABBREVIATIONS, ACRONYMS AND SYMBOLS

™ - Trademark

® - Registered trademark

ADAM – A desintegrin and metalloproteinase

AG – Adelina Gama

ALCAM – Activated leucocyte cell adhesion molecule

AMG – Adjacent mammary gland

Anapl. – Anaplastic

AR – Androgen receptor

Bcl-2 - B-cell lymphoma 2

CA – Complex Adenoma

CAMs – Cell adhesion molecules

Carc. – Carcinoma

COX-2 – Cyclo-oxygenase 2

CRYAB – αB-Crystallin

DAB – 3,3'-diaminobenzidine tetrahydrochloride

ECM – Extracellular matrix

ECs – Endothelial cells

EGFR – Epidermal growth factor receptor

EMT- Epithelial to mesenchymal transition

ER – Estrogen receptor

ERK – Extracellular signal-regulated kinases

FAK – Focal adhesion kinase

FGFR – Fibroblast growth factor receptor

FM – Francisco Mendes

GS – Global Score

HE – Hematoxylin and eosin

HPF – High power fields

Hsps – Heat shock proteins

ICD – Intracellular domain

Ig – Immunoglobulin

IMC – Inflammatory mammary cancer

INF-ɣ – Gamma interferon

kDa- kiloDalton

L1CAM – L1 Cell adhesion molecule

LP – Laura Peña

MAPK – Mitogen-activated protein kinases

MBT – Mixed benign tumor

Myoep. – Myoepithelioma

N-CAM – Neural cell adhesion molecule

NF-1A – Nuclear factor 1A

NF-κB – Nuclear factor kappa B

NIMC – Non-inflammatory mammary cancer

OHE - Ovariohysterectomy

PBS – Phosphate Buffered Saline

PR – Progesterone receptor

PS – Percentage of stain

REST – RE1 Silencing transcription factor

RGD-motif – Arginyl-glycyl-aspartic acid motif

RNA - Ribonucleic acid

sHsps – Small heat shock proteins superfamily

SI – Staining intensity

Src – Proto-oncogene tyrosine-protein kinase Src

TAG-1 – Transient axonal glycoprotein 1

TGF-β1 – Transforming growth factor betta 1

TNF-α – Tumor necrosis factor alpha

TNM – Method of classification of malignant tumors: T-Tumor; N-Lymph Node; M-Metastasis

TRAIL - TNF-related apoptosis-inducing ligand

TS – Total score

UCM – Universidade Complutense de Madrid (Complutense University of Madrid)

UV – Ultraviolet

VEGF-A – Vascular endothelial growth factor A

WHO – World Health Organization

1. INTRODUCTION

Canine mammary tumors (CMTs) are the most common neoplasm found in intact female dogs, representing around 50% of all tumors diagnosed (Merlo et al., 2008; Vascellari et al., 2009; Sleeckx et al., 2011; Salas et al., 2015). Around 50% of these are malignant, being most of them of epithelial origin. This makes carcinomas the most frequent malignant mammary neoplasms diagnosed in female dogs, with sarcomas (malignant mesenchymal tumors) representing less than 5% of the diagnosed neoplasms (Sleeckx et al., 2011). Lately, the incidence of CMTs is decreasing in countries where ovariohysterectomy (OHE) is being performed at an early age as a preventive measure to avoid CMTs (Sleeckx et al., 2011).

Histopathological classification of CMTs is highly important, since different histological subtypes may have a different prognosis. For instance, an anaplastic carcinoma has the worst prognosis of all subtypes of CMTs and a complex carcinoma is usually associated with a good prognosis, when we are strictly talking about histopathological classification by itself (Goldschmidt et al., 2011). Nowadays, the most globally accepted classification system to CMTs was published in 1999 by the World Health Organization. Since then, research in CMTs has been arising interest and numerous investigations have increased the knowledge in this area. With that in mind, Goldschmidt et al. (2011) have proposed a new classification system, introducing new subtypes of CMTs. Throughout this dissertation, this updated classification will be used to classify CMTs.

Besides classification, grading of malignant CMTs is also of high importance, since it can influence prognosis and treatment protocols. The grading system relies in 3 major features of malignancy: tubule formation, nuclear pleomorphism and number of mitoses per 10 high power fields (HPF). These features are classified from 1 to 3 points and then summed up to obtain a total score (from 3 to 9 points). The higher the total score, the higher the grade and, consequently, the higher the malignancy (Peña et al., 2012).

CMTs are now divided in 8 major categories: malignant epithelial neoplasms (carcinomas), special types of carcinomas, malignant mesenchymal neoplasms (sarcomas), malignant mixed mammary tumors (carcinosarcomas), benign neoplasms, hyperplasia or dysplasia, neoplasms of the nipple and hyperplasia/dysplasia of the nipple (Goldschmidt et al., 2011). Sometimes, histologic evaluation by itself is not enough to distinguish between different types of tumors and immunohistochemistry must be performed to classify the CMT(Goldschmidt et al., 2011; Peña et al., 2014).

Risk factors to develop CMTs include age, breed, genetics, hormones, diet, among others (Sleeckx et al., 2011). Older animals have an increased risk of developing CMTs, probably

because of their longer exposition to the ovarian hormones that are, by themselves, a risk factor too. These hormones stimulate the growth of the mammary tissue under physiologic conditions (steroids induce epithelial proliferation), and they probably also induce tumorigenic growth. Since this hormonal exposure occurs in each oestrus, it is easily understandable that female dogs tend to get more sensitive to this exposure as they get older. Knowing this, preventive spaying at an early age is a key factor to fight the high prevalence of CMTs. It is now known that female dogs spayed before the first oestrus have a risk of 0,5% of developing CMTs, while dogs spayed before the second oestrus or after the second oestrus have a risk of 8 or 26% of developing CMTs, respectively (Sleeckx et al., 2011). Breed or genetic predisposition is also thought to influence the development of CMTs, but studies are contradictory. OHE by the time of tumor removal was found to have positive influence in the prognosis of dogs with estrogen receptor positive mammary carcinomas, grade 2 CMT's or with increased peri-surgical serum E2 concentration (Kristiansen et al., 2016).

Treatment of CMTs usually implicates surgery (except those cases of inflammatory carcinoma, where surgery is not indicated) and can involve post-surgical chemotherapy (Sleeckx et al., 2011; Tran et al., 2016). Radiotherapy, hormonal therapy, antiangiogenic strategies, among others, are not routine treatment protocols for CMTs, and surgery remains the gold standard of treatment, especially in early stage, low grade tumors (Sleeckx et al., 2011). Other treatment protocols are more useful in malignant cases, when metastasis are present, or in which surgery cannot be performed, either by anesthetic precautions or by surgical planning (Sleeckx et al., 2011; Tran et al., 2016).

Mechanisms responsible for the conversion of a regular cell into a cancerous one are very complex but have been subject of deep investigation from cancer researchers. We are now able to identify 6 major key events/features that a cell needs to achieve malignancy. These 6 features (summarized in Figure 1) represent the result of years of cancer investigation, either in humans, animals and *in vitro,* and resume all the features that researchers believe that are responsible for the tumor formation and proliferation (Hanahan & Weinberg, 2000). These features are the following, including some examples of each of them (Hanahan & Weinberg, 2000):

1. Self-sufficiency in growth signals – The overexpression of HER-2 in mammary carcinomas;
2. Insensitivity to anti-growth signals – The phosphorylation of the retinoblastoma protein, suppressor of growth;
3. Evading apoptosis – The inactivation of the gene *p53* which is a tumor suppressor;
4. Limitless replicative potential – Activation of telomerase;

5. Tissue invasion/metastasis – Changing properties of the cell-cell adhesion molecules (CAM's) and integrins (cell-extracellular matrix adhesion);
6. Sustained angiogenesis – Increased expression of VEGF.

More recently two more features have been proposed, and are now classified as "emerging hallmarks", until further prove is acquire that they are transversal to almost every type of cancer, such as the previous six. These two new features are (a) the capability of a cell to deregulate the cell metabolism and energetics and (b) to avoid destruction by the immune system (Hanahan & Weinberg, 2011).

Each of these features represents a further step in the pathway to the cancerous capacity of cells, which is believed to happen in all (or almost all) types of cancer. At the molecular level, these features are way too complex to be the subject of study on this dissertation. Since we could not talk about all of these, and considering the material we had in our power, we decided to investigate two different molecules that could fit in some of the categories of the *hallmarks of cancer*.

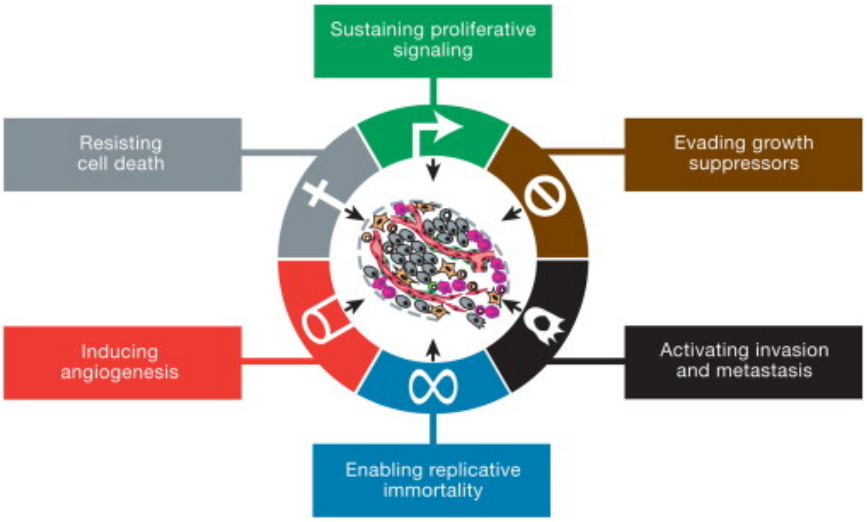

Figure 1 – The Hallmarks of Cancer (Hanahan & Weinberg, 2011)

αB-Crystallin (CRYAB) is a member of the small heat chock protein superfamily (sHsps), which is a group of molecules responsible for the maintenance of the cellular homeostasis (Sun & MacRae, 2005). It is expressed in response to multiple stress factors including heat shock, cytokines, and others (Ilhan et al., 2010; Guvenc et al., 2012). It was considered as a marker of poor prognosis (Moyano et al., 2006), as well as a marker for basal-type and metaplastic breast carcinomas in women (Moyano et al., 2006; Sitterding et al., 2008; Chan et al., 2011).

L1 cell adhesion molecule (L1CAM) is a transmembrane glycoprotein involved in cell-cell interaction as well as cell to extracellular matrix interactions (Homrich et al., 2015; Samatov et al., 2016) discovered in mice brain in 1984 (Rathjen & Schachner, 1984). Its expression is regulated by a variety of genes namely RE1-Silencing Transcription factor (REST) (Kiefel et al., 2012; Samatov et al., 2016) and others. Overexpression of this molecule has been correlated to poor prognosis, chemotherapy resistance, shorter disease-free interval, high grades of malignancy, epithelial to mesenchymal transition (EMT), among others (Kiefel et al., 2012; Kiefel et al., 2012; Zhang et al., 2015; Altevogt et al., 2016; Samatov et al., 2016).

2. ALPHA B-CRYSTALLIN

2.1. Review

2.1.1. Structure

αB-Crystallin (CRYAB) is a heat shock protein, belonging to the small heat shock proteins superfamily (sHsps). The heat shock proteins are divided in 6 major families according to their molecular weight, being those the Hsp 100, Hsp 90, Hsp 70, Hsp 60, Hsp 40 and, for last, the small heat shock proteins (Bakthisaran et al., 2015). The latter represent a group of molecules responsible for the maintenance of the cellular homeostasis (Sun & MacRae, 2005), preventing cell damage and promoting cell survival (Clark & Muchowski, 2000; Moyano et al., 2006). CRYAB belongs to a protein family composed by three classes, including alpha, beta and gamma crystallins. The group of alpha crystallins includes both acid and basic crystallins, giving rise to either αA-Crystallin or αB-Crystallin, respectively (Ilhan et al., 2010). Composed by 175 amino acids residues (Wistow, 1985), CRYAB has a molecular weight of 22 kDa, but its tridimensional structure is not well defined yet (Ilhan et al., 2010). However, sHsp are in general composed by a conserved α-crystallin core domain, a N-terminal domain and a C-terminal extension domain, with typical weights between 12 and 43 kDa (Ghosh et al., 2005; Bakthisaran et al., 2015; Tikhomirova et al., 2017).

CRYAB is expressed in response to multiple stress factors including heat shock, cytokines, and others (Ilhan et al., 2010; Guvenc et al., 2012). It is considered as a marker of poor prognosis in breast cancer (Moyano et al., 2006), as well as a marker for basal-type and metaplastic breast carcinomas in women (Moyano et al., 2006; Sitterding et al., 2008; Chan et al., 2011).

2.1.2. Expression

First reported in the human lens (Delaye & Tardieu, 1983), where it is responsible for maintaining the lens transparency (Delaye & Tardieu, 1983), it was readily proved that its expression was extended to the heart, brain, spleen, kidney, lung and almost all vertebrate cells (Dubin et al., 1989). In humans, the gene encoding CRYAB is located on chromosome 11 (Ngo et al., 1989).

In human breast, αB-Crystallin was shown to be expressed by the myoepithelial cells in normal tissue samples, proliferative diseases and myoepithelial cells surrounding the area of *in situ* carcinomas (Moyano et al., 2006; Sitterding et al., 2008). It is also expressed by adipocytes (Moyano et al., 2006; Sitterding et al., 2008; Chan et al., 2011) and the vascular smooth muscle wall (Chan et al., 2011). Luminal epithelium of breast samples was always negative in these

situations (Moyano et al., 2006; Sitterding et al., 2008; Chan et al., 2011). Expression by tumor cells is further discussed below.

2.1.3. Interactions/function

In human medicine, the small heat shock proteins are involved in cataracts formation, desmin-related myopathy, neurological diseases (such as Alzheimer, Parkinson or Huntington's Syndrome), and cancer formation/progression (Sun & MacRae, 2005).

Regarding αB-Crystallin, it is responsible for maintaining cellular homeostasis by acting as a molecular chaperone (class of proteins responsible for maintaining the original structure of cell proteins (Tikhomirova et al., 2017)), preventing protein aggregation/denaturation and consequent cellular damage. This type of damage occurs because of the sHsps response to oxidative stress, heat shock or other stress factors (Sun & MacRae, 2005; Ilhan et al., 2010; Guvenc et al., 2012; Bakthisaran et al., 2015; Tikhomirova et al., 2017). It is also responsible for conceding antiapoptotic features to cells (Malin et al., 2016).

As stated before, CRYAB is responsible for maintaining the lens transparency, since it avoids protein aggregates formation and inhibits apoptosis and consequent protein degradation in the lens. It is also reported as having an important role in maintaining the heart muscle functional integrity and preserving its contractility. In the brain, its overexpression is related to neurodegenerative diseases such as Alzheimer, Parkinson, lateral amyotrophic sclerosis and Alexander's disease, but its role in these diseases is not the scope of the present dissertation. As a curiosity, CRYAB was overexpressed in the brain of patients with Alzheimer, and an association was found between CRYAB and the deposit of the amyloid plaques in astrocytes, a characteristic feature of this disease (Boelens, 2014).

It was linked to the inhibition of the myogenic apoptosis through inhibition of the protease caspase-3 (Kamradt et al., 2002). This finding is consistent with the findings of the same authors regarding CRYAB expression in cancer cell lines, as discussed on 2.1.4. (Kamradt et al., 2005).

Moyano et al. (2006) proved that the overexpression of αB-Crystallin was responsible for augmenting the tumor invasiveness and cell motility *in vitro*, as well as in promoting the emergence of mammary carcinomas in nude mice, *in vivo*. The first was further verified to be dependent of the activation of the MAPK/ERK pathway, since the blockage of this pathway highly inhibited motility and invasiveness of tumor cells. RNA silencing of αB-Crystallin also inhibited the phenotypical changes responsible for this increased motility and invasiveness.

2.1.4. αB-Crystallin in cancer

Hsps are overexpressed in a variety of tumors, and their role in tumor development has been linked to both the promotion of autonomous cell proliferation and inhibition of mechanisms of cellular death (reviewed by Calderwood et al., 2006). Although this review does not encompass our marker, CRYAB, it does show the important role of Hsps, in general, in cancer progression. Their function in breast cancer has been reported both in women and in female dogs back in 2005, where a variety of markers were studied in breast/mammary tumors, including the HSPs 90 and 70, caspase 3 and 8, Bcl-2 and others. The authors proved that dysbalanced antiapoptotic and proapoptotic features, resulting in cellular death "escape" from cancer cells represents an important step in malignancy (Kumaraguruparan et al., 2005), as stated in the world acclaimed article "*The hallmarks of cancer*" (Hanahan & Weinberg, 2000).

With regard to CRYAB, several authors have described its overexpression in a variety of tumors, such as renal carcinomas (Pinder et al., 1994), breast carcinomas (Chelouche-Lev et al., 2004), gliomas and others (reviewed by Gruvberger-Saal et al., 2006). Authors do not believe that its role in cancer is due to a mutation in the gene that regulates its expression. Instead, it's thought that the response of Hsps (including CRYAB) to multiple stress factors results in an overexpression, which will represent a key factor in tumor development, aggressiveness and, consequently, prognosis (Malin et al., 2016).

It was also proved that CRYAB could inhibit apoptosis in certain cell lines. First, it was reported that it was able to inhibit the Tumor Necrosis Factor alpha (TNFα) cytokine, through inhibition of caspase 3 (Kamradt et al., 2001). Further, it was reported that the same happens in myogenesis, during myoblasts differentiation into myocytes (Kamradt et al., 2002) and finally it was proved that the previously reported inhibition of caspase-3 happens through the TNF-related apoptosis-inducing ligand (TRAIL) (Kamradt et al., 2005). TRAIL is a member of the TNFα family, known for selectively inducing apoptosis of cancer cells, but preserving the normal ones, making it a promising cancer therapeutic agent. The same authors proved, for the first time, that CRYAB could actually promote tumor growth *in vivo* (Kamradt et al., 2005) when they tested mice baring anti-apoptotic CRYAB activity (using a CRYAB mutant), which had significantly reduced tumor growth when compared to athymic nude mice with wild type CRYAB.

Presently, it is known that CRYAB is capable of inhibiting apoptosis either by interfering with the intrinsic/mitochondrial or with the extrinsic/death receptor pathway. Both pathways culminate in activation of caspase-3, either by caspase 9 or caspase 8, respectively (Malin et al., 2016). CRYAB is capable of inhibiting these pathways, resulting in no activation of caspase 3,

which leads to a resistance to multiple apoptotic stimuli, such as the TNFα, TRAIL, hypoxia, chemotherapy agents, UV radiation, among others (Malin et al., 2016).

In a recent review, CRYAB was described as a metastasis enabler, by a proposed multifactorial role in cancer development. The authors concluded that CRYAB has a role in angiogenesis, EMT, extravasation, apoptosis resistance, migration, invasion, among other important factors in metastasis formation (Malin et al., 2016).

In breast cancer, CRYAB has shown to be an excellent marker for basal-like breast tumors and for metaplastic carcinomas, which are characterized by an overexpression when compared to other types of breast cancer (Moyano et al., 2006; Sitterding et al., 2008; Chan et al., 2011). Basal-like breast tumors are commonly referred to as triple negative breast tumors, since they usually lack expression of ER, PR and HER-2 (Reis-Filho et al., 2006) and express basal markers, such as cytokeratin 5 and 14 and p63, characteristic of myoepithelial/basal cells, such as in canines (Peña et al., 2014). Metaplastic carcinomas are a rare form of breast carcinomas, characterized by having spindle and/or metaplastic features and are believed to be a subgroup of basal-like tumors (Reis-Filho et al., 2006).

To our knowledge, there is only one article regarding canine mammary cancer and CRYAB, which refers its expression in luminal epithelial cells and not in the myoepithelium, contrarily to the previously reported findings in normal human breast samples. They also concluded that the higher the malignancy of the tumor, the higher the expression of the protein, by comparing hyperplastic tissue, benign and malignant tumors expression, which made the authors link this marker with poor prognosis (Guvenc et al., 2012).

2.1.5. αB-Crystallin as a marker of prognosis

In terms of prognosis, the protein is linked to poor clinical outcome and shorter patient survival in breast cancer (Moyano et al., 2006; Sitterding et al., 2008) since its expression is linked to higher tumor growth and chemotherapy resistance (Kamradt et al., 2001, 2002, 2005). In basal-like breast tumors, CRYAB was proved to be an independent prognostic factor (Moyano et al., 2006). It was also tested as a marker for lymph node involvement in breast carcinomas, where a strong correlation was found, giving rise to the hypothesis that CRYAB can be a marker of breast cancer progression and prognosis (Chelouche-Lev et al., 2004). An association between brain metastasis formation and CRYAB overexpression in breast cancer was also found (Malin et al., 2014), as well as an association with chemotherapy resistance (Ivanov et al., 2008) and tumor growth (Kamradt et al., 2005).

2.2. Objectives

The goals of our study were:

- to evaluate the immunohistochemical expression of CRYAB in a series of canine mammary gland tissues;

- to determine an association between its expression with clinicopathological parameters, namely between different histological groups.

2.3. Material and Methods

2.3.1. Tumor specimens

Canine mammary gland tumor specimens were obtained from the archives of the Histopathology Laboratory of the University of Trás-os-Montes and Alto Douro, Vila Real. Tumor samples were surgically removed from 79 female dogs by lumpectomy or mastectomy (regional or radical). From the available archival material obtained between 1999 and 2015, selected normal/hyperplastic (n=9), benign (n=15) and malignant (n=55) mammary tumors were studied. The material had been fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin wax. Sections (3 μm) were cut for histological examination and for immunohistochemistry.

2.3.2. Clinicopathological parameters evaluation

Clinical data included animal breed, age, reproductive status (intact/ ovariohysterectomized with mastectomy or prior to tumor development), previous administration of oestrus-prevention medications and tumor characteristics (location, size, skin ulceration). Tumor size was defined as the maximum diameter, with tumors grouped according to the TNM World Health Organization (WHO) staging of canine mammary tumors in: tumors with less than 3 cm; tumors with 3-5 cm and tumors larger than 5 cm (Rutteman et al., 2001).

All tumor samples were revised in haematoxylin and eosin (HE) stained sections, according to the new proposed classification for canine mammary neoplasms (Goldschmidt et al., 2011). Other histopathological parameters evaluated included: histological grade, lymphovascular invasion (presence *vs.* absence) and lymph node metastases (presence *vs.* absence).

Histological grade was evaluated in malignant neoplasms, according to the method for canine mammary tumors (Goldschmidt et al., 2011), which is based on Elston and Ellis (1988) criteria by the assessment of three morphological features: tubule formation, nuclear pleomorphism and mitotic counts. Each of these features was scored as 1, 2 or 3 to indicate whether it was present in slight, moderate or marked degree, respectively, giving a putative total of 3-9 points. Grade was allocated by an arbitrary division of the total points as follows: grade I (well differentiated), 3, 4 or 5 points; grade II (moderately differentiated), 6 or 7 points; and grade III (poorly differentiated), 8 or 9 points. Mitotic counts were assessed as the number of mitoses per 10 high power fields (40x) at the most mitotically active areas.

For the grading and immunolabeling quantification, a Nikon Labophot microscope was used (HPF area=0,152 mm^2).

2.3.3. Immunohistochemistry

Immunohistochemistry was performed with a mouse monoclonal antibody raised against αB-crystallin (Clone 1B6.1-3G4, 1:200, Stressgen Biotechnologies/Enzo Life Sciences).

Slides were deparaffinized for 30 minutes and then hydrated with solutions of alcohol with consecutively higher concentrations (70%, 80%, 90% and finally 100%), for 5 minutes. Antigen retrieval was carried out by microwave treatment in 10 mM citrate buffer, pH 6.0, in 3 cycles of 5 minutes. It was then chilled down for 30 minutes at room temperature. Slides were washed with Phosphate Buffered Saline (PBS) and endogenous peroxidases were blocked by a 3% Hydrogen Peroxide solution for 30 minutes. After another wash with PBS, protein blocking was performed using the Novacastra™ Protein Block solution for 5 minutes. Primary antibody was added (concentration 1:200) and incubated overnight in a humid chamber at 4ºC. After washing with PBS, a polymeric labeling methodology was used as a detection system (Novolink Polymer Detection System, Leica Biosystems®, Newcastle, United Kingdom), following the manufacturer's instructions. Briefly, slides were incubated for 30 minutes with the Post-Primary solution, followed by another 30 minutes incubation with the Polymer solution. Washing with PBS was performed between these 2 steps. Finally, the color was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and slides were counterstained with Gill's hematoxylin, dehydrated, and mounted for evaluation by light microscopy.

2.3.4. Quantification of immunolabeling

Quantification of immunolabeling was performed by two observers (FM and AG). To evaluate CRYAB expression in canine mammary tissues we adapted the method used by Kim et al. (2015). We classified the stain intensity (SI) in 0, 1, 2 or 3 corresponding to no stain, weak, moderate or strong epithelial staining, respectively. We also evaluated the percentage of staining (PS), which was divided in 5 categories: 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%) (Kim et al., 2015). A total score (TS) was obtained, by multiplying the SI for the PS (TS= SI*PS), with total scores ranging from 0 to 12. Finally, we defined two major categories: low score, for values from 0 to 3, and high score, for values of 4-12. This last division was denominated as Global score (GS) (Table 1).

Table 1 - Quantification of CRYAB immunolabeling

Stain Intensity (SI)	Percentage of Staining (PS)	Total Score (TS=SI*PS)	Global Score (GS)
0 (Absent)	0 (0%)	0 to 12	Low (0-3) High (4-12)
1 (Weak)	1 (1-25%)		
2 (Moderate)	2 (26-50%)		
3 (Strong)	3 (51-75%)		
	4 (75-100%)		

2.3.5. Statistical analysis

To the comparative study of CRYAB immunolabeling with the multiple variables, the Pearson's Chi-Square and Fisher's exact test were used when appropriate. Analysis was performed by IBM SPSS Statistics 24 software, with p values < 0.05 considered statistically significant.

2.4. Results

2.4.1. Clinicopathological characteristics

The clinicopathological characteristics of the present series and respective frequencies are presented on Table 2.

Animal age (n=70) ranged between 4 and 16 years (medium = 9.79 ± 2.62 years old). Tumor size (n=65) varied between 0,4 cm and 20 cm (medium = 4,63 ± 4.15 cm). Most of the tumors were localized on the most caudal mammary glands (M4 and/or M5), representing 47.2% (25/53) of the samples. Several breeds were represented in our study, with Poodle and Cocker Spaniels being the more representative ones; however, female dogs of undetermined breed were the most common (n=35; 47.9%). Most tumors were not ulcerated (n=55; 80.9%) and had less than 3 cm of diameter (n=27; 41.5%); however, tumors larger than 5 cm in diameter did represent a significant part of the samples studied (n=24; 36.9%). Most animals were not submitted to ovariohysterectomy (n=38; 79.2%), and did not receive contraceptive drugs (n=29; 76.3%).

Malignant neoplasms were the most represented group (n=55; 69.6%), with most carcinomas classified as grade 3 (poorly differentiated) tumors (n=22; 40.0%). Most of the cases were already metastatic to the lymph nodes (n=17; 65.4%) and vascular invasion was present in nearly 50% of the cases (n=28).

Table 2 - Clinicopathological characteristics of the present series.

Clinicopathological Characteristics	n (%)
Age (n=70)	
<10 years	34 (48.6%)
≥10 years	36 (51.4%)
Total	70 (100%)
Breed (n=73)	
Undetermined	35 (47.9%)
Poodle	10 (13.7%)
Cocker Spaniel	7 (9.6%)
Others	6 (28.8%)
Total	73 (100%)
Tumor Size (n=65)	
<3 cm	27 (41.5%)
3-5 cm	14 (21.5%)
>5 cm	24 (36.9%)
Total	65 (100%)
Location (n=53)	
M1 and/or M2	7 (13.2%)
M3	13 (24.5%)
M4 and/or M5	25 (47.2%)
Multiple	8 (15.1%)
Total	53 (100%)
Ulceration (n=68)	
Absent	55 (80.9%)
Present	13 (19.1%)
Total	68 (100%)
OHE (n=48)	
Not performed	38 (79.2%)
Performed	10 (20.8%)
Total	48 (100%)
Contraception (n=38)	
Not performed	29 (76.3%)
Performed	9 (23.7%)
Total	38 (100%)
Histological group (n=79)	
Hyperplasic/normal mammary gland	9 (11.4%)
Benign neoplasm	15 (19.0%)
Malignant neoplasm	55 (69.6%)
Total	79 (100%)
Histological grade (n=55)	
1	16 (29.1%)
2	17 (30.9%)
3	22 (40.0%)
Total	55 (100%)
Vascular invasion (n=55)	
Absent	28 (50.9%)
Present	27 (49.1%)
Total	54 (100%)
Lymph node metastasis (n=26)	
Absent	9 (34.6%)
Present	17(65.4%)
Total	26 (100%)

The histological diagnosis of tumor samples is presented in Table 3. The most common neoplasm was the tubulopapillary carcinoma (n=15; 19.0%), being also the most common within the malignant type. The most common benign neoplasm was the complex adenoma (n=11; 13.9%).

Table 3 - Histological diagnosis and respective frequencies

Histological diagnosis	n (%)
Hyperplastic mammary gland (n=6)	6 (7.6%)
Benign tumors (n=15)	
Ductal Adenoma	1 (1.3%)
Intraductal papilloma	1 (1.3%)
Benign mixed tumor	2 (2.5%)
Complex Adenoma	11 (13.9%)
Malignant tumors (n=55)	
Anaplastic Carcinoma	1 (1.3%)
Malignant myoepithelioma	1 (1.3%)
Carcinosarcoma	4 (5.1%)
Complex Carcinoma	5 (6.3%)
Carcinoma and malignant myoepithelioma	8 (10.1%)
Carcinoma in Complex Adenoma/Benign mixed tumor	9 (11.4%)
Solid Carcinoma	12 (15.2%)
Tubulopapillary Carcinoma	15 (19.0%)
Total	76 (100%)

2.4.2. Immunolabeling of CRYAB in the normal mammary gland and hyperplasia

With regard to the immunolabeling of the normal and hyperplasic mammary gland (n=3 and n=6, respectively), the results are described on Table 4.

CRYAB staining in normal/hyperplastic glands was mainly observed in luminal epithelial cells, with focal myoepithelial positivity. Normal mammary gland tends to obtain a low GS score (n=2; 66.7%), usually associated with week staining intensity, while hyperplastic mammary gland

had a 50% equal distribution in terms of GS, with strong intensity representing the most common SI (n=3; 50%) (Fig. 2a). Regarding PS, the normal tissue usually showed 26-50% of positive cells (n=2; 66.7%), while hyperplastic mammary gland was more frequently characterized by less than 25% of stained cells (n=3; 50.0%), with a heterogeneous pattern of expression. Ductal hyperplasia (epitheliosis) was usually strongly positive. No significant differences were found between normal and hyperplastic glands (p=1). Consistently, CRYAB expression was also observed in muscle, nerves and vessels.

Table 4 - Immunolabeling of CRYAB in normal/hyperplastic mammary gland

CRYAB immunoexpression		Normal mammary gland n(%)	Hyperplastic mammary gland n(%)
Staining Intensity	**Absent**	0 (0%)	0 (0%)
	Weak	2 (66.7%)	2 (33.3%)
	Moderate	1 (33.3%)	1 (16.7%)
	Strong	0 (0%)	3 (50.0%)
Percentage of Staining	**0%**	0 (0%)	0 (0%)
	1-25%	1(33.3%)	3 (50.0%)
	26-50%	2 (66.7%)	1 (16.7%)
	51-75%	0 (0%)	2 (33.3%)
	76-100%	0 (0%)	0 (0%)
Global Score	**Low**	2 (66.7%)	3 (50.0%)
	High	1 (33.3%)	3 (50.0%)
	Total	3 (100%)	6 (100%)

2.4.3. Immunolabeling of CRYAB in benign neoplasms

The CRYAB immunolabeling of benign neoplasms is presented on Table 5. All benign samples analysed were positive, with a predominance of moderate intensity (n=8; 53.3%) and a percentage of more than 75% (n=9; 60.0%). Consequently, GS was usually high (n=13; 86.67%).

Complex adenomas always obtained high GS (n=11; 100%) (Fig. 2b), as well as mixed benign tumors (n=2; 100%). Ductal adenoma and intraductal papilloma were classified as low GS. Although few cases were analysed, a p value of 0.019 regarding GS revealed that these

differences are statistically significant. CRYAB expression was observed in both neoplastic luminal epithelial and myoepithelial cells, with these ones usually showing strong intensity and high percentage of staining in the complex adenoma and mixed benign tumor cases.

Table 5 - Immunolabeling of CRYAB in benign neoplasms

CRYAB immunolabeling		Complex Adenoma n (%)	Ductal Adenoma n (%)	Intraductal papilloma n (%)	Mixed Benign Tumor n (%)
SI	Absent	0 (0%)	0(0%)	0 (0%)	0 (0%)
	Weak	0 (0%)	1 (100%)	1 (100%)	0 (0%)
	Moderate	6 (54.5%)	0 (0%)	0 (0%)	2 (100%)
	Strong	5 (45.5%)	0 (0%)	0 (0%)	0 (0%)
PS	0%	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	1-25%	0 (0%)	1 (100%)	1 (100%)	0 (0%)
	26-50%	1 (9.1%)	0 (0%)	0 (0%)	0(0%)
	51-75%	2 (18.2%)	0 (0%)	0 (0%)	1 (50.0%)
	76-100%	8 (72.7%)	0 (0%)	0 (0%)	1 (50.0%)
GS	Low	0 (0%)	1 (100%)	1 (100%)	0 (0%)
	High	11 (100%)	0 (0%)	0 (0%)	2 (100%)
	Total	11 (100%)	1 (100%)	1 (100%)	2 (100%)

2.4.4. Immunolabeling of CRYAB in malignant neoplasms

The results regarding CRYAB expression in malignant tumors are shown on Table 6. Out of the 55 malignant neoplasms available, 34 of them showed moderate SI (61.8%) and 26 showed 1 to 25% of PS (47.3%); thus, the GS was mostly low (n=29; 52.7%).

Of note, only two carcinomas were completely negative (tubulopapillary carcinoma subtype), with simple carcinomas (anaplastic carcinoma, solid carcinoma and tubulopapillary carcinoma) predominantly characterized by a low GS (Fig. 2c). In contrast, non-simple carcinomas (complex carcinoma, carcinoma and malignant myoepithelioma and carcinoma in CA/MBT) and the only analysed malignant myoepithelioma obtained a high GS, with both epithelial and myoepithelial neoplastic cells positive (Fig. 2d). A *p* value of 0.039 was found regarding the GS, meaning significant differences were found between different histological types.

Table 6 - Immunolabeling of CRYAB in malignant neoplasms

CRYAB		Anapl carc.	Complex carc.	Carc. and malignant myoep.	Carc. in CA/MBT	Solid carc.	Tubulo-papillary carc.	Carcino sarcoma	Malignant myoep.
SI	**Absent**	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (13.3%)	0 (0%)	0 (0%)
	Weak	1 (100%)	0 (0%)	0 (0%)	2 (22.2%)	1 (8.3%)	2 (13.3%)	1 (25.0%)	0 (0%)
	Moderate	0 (0%)	5 (100%)	5 (62.5%)	4 (44.4%)	7 (58.3%)	11 (73.3%)	1 (25.0%)	1 (100%)
	Strong	0 (0%)	0 (0%)	3 (37.5%)	3 (33.3%)	4 (33.3%)	0 (0%)	2 (50.0%)	0 (0%)
PS	**0%**	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (13.3%)	0 (0%)	0 (0%)
	1-25%	1 (100%)	1 (20.0%)	2 (25.0%)	2 (22.2%)	7 (58.3%)	10 (66.7%)	3 (75.0%)	0 (0%)
	26-50%	0 (0%)	1 (20.0%)	1 (12.5%)	2 (22.2%)	3 (25.0%)	2 (13.3%)	0 (0%)	0 (0%)
	51-75%	0 (0%)	1 (20.0%)	4 (50.0%)	4 (44.4%)	1 (8.3%)	1 (6.7%)	1 (25.0%)	0 (0%)
	76-100%	0 (0%)	2 (40.0%)	1 (12.5%)	1 (11.1%)	1 (8.3%)	0 (0%)	0 (0%)	1 (100%)
GS	**Low**	1 (100%)	1 (20.0%)	2 (25.0%)	3 (33.3%)	7 (58.3%)	12 (80.0%)	3 (75.0%)	0 (0%)
	High	0 (0%)	4 (80.0%)	6 (75.0%)	6 (66.7%)	5 (41.7%)	3 (20.0%)	1 (25%)	1 (100%)

2.4.5. Comparative study of CRYAB immunoexpression with the clinicopathological characteristics

When comparing CRYAB GS with the clinicopathological characteristics, significant differences were found in tumor size (p=0.039), histological group (p=0.022) and presence of lymph node metastasis (p=0.014). Larger tumors were usually characterized by a low GS (n=14; 58.3%). With regard to the histological group, benign neoplasms showed higher scores (n=13; 86.7%), while normal/hyperplastic and malignant neoplasms tended to obtain lower scores (n=5; 55.6% and n=29; 52.7%, respectively). Regarding metastasis, CRYAB GS was higher (n=7; 77.8%) in cases with no lymph node metastasis. When present, the score tended to be low (n=13; 76.5%). These results are shown on Tables 7 and 8.

Table 7 - Comparative study of CRYAB GS with the clinical characteristics

Clinical Characteristics	CRYAB GS n (%)		*p* value
	Low	High	
Age (n=70)			
<10 years (n=34)	15 (44.1%)	19 (55.9%)	0.641
≥10 years(n=36)	18 (50.0%)	18 (50.0%)	
Total	33 (47.1%)	37 (52.9%)	
Breed (n=73)			
Indetermin. (n=35)	17 (48.6%)	18 (51.4%)	
Poodle (n=10)	3 (30.0%)	7 (70.0%)	0.279
Cocker Spaniel (n=7)	2 (28.6%)	5 (71.4%)	
Others (n=21)	13 (61.9%)	8 (38.1%)	
Total	35 (47.9%)	38 (52.1%)	
Tumor Size (n=65)			
<3 cm (n=27)	7 (25.9%)	20 (74.1%)	
3-5 cm (n=14)	8 (57.1%)	6 (42.9%)	**0.039**
>5 cm (n=24)	14 (58.3%)	10 (41.7%)	
Total	29 (44.6%)	36 (55.4%)	
Location (n=53)			
M1 and/or M2 (n=7)	3 (42.9%)	4 (57.1%)	
M3 (n=13)	5 (38.5%)	8 (61.5%)	
M4 and/or M5 (n=25)	11 (44.0%)	14 (56.0%)	0.976
Multiple (n=8)	4 (50.0%)	4 (50.0%)	
Total	23 (43.4%)	30 (56.6%)	
Ulceration (n=68)			
Absent (n=55)	21 (38.2%)	34 (61.8%)	0.211
Present (n=13)	8 (61.5%)	5 (38.5%)	
Total	29 (42.6%)	39 (57.4%)	
OHE (n=48)			
Not performed (n=38)	21 (55.3%)	17 (44.7%)	1.0
Performed (n=10)	5 (50.0%)	5 (50.0%)	
Total	26 (54.2%)	22 (45.8%)	
Contraception (n=38)			
Not performed (n=29)	14 (48.3%)	15 (51.7%)	0.254
Performed (n=9)	2 (22.2%)	7 (77.8%)	
Total	16 (42.1%)	22 (57.9%)	

No significant differences were found for other clinicopathological parameters. However, for vascular invasion, there was a tendency to carcinomas with vascular invasion exhibiting a low GS (n=18; 66.7%)

Table 8 - Comparative study of CRYAB GS with the histopathological characteristics

Pathological Characteristics	CRYAB GS n (%)		*p* value
	Low	High	
Histological group (n=79)			
Hyperplasic/normal mammary gland (n=9)	5 (55.6%)	4 (44.4%)	**0.022**
Benign neoplasm (n=15)	2 (13.3%)	13 (86.7%)	
Malignant neoplasm (n=55)	29 (52.7%)	26 (47.3%)	
Total	36 (45.6%)	43 (54.4%)	
Histological grade (n=55)			
1 (n=16)	7 (43.8%)	9 (56.3%)	0.187
2 (n=17)	7 (41.2%)	10 (58.8%)	
3 (n=22)	15 (68.2%)	7 (31.8%)	
Total	29 (52.7%)	26 (47.3%)	
Vascular invasion (n=55)			
Absent (n=28)	11 (39.3%)	17 (60.7%)	0.060
Present (n=27)	18 (66.7%)	9 (33.3%)	
Total	29 (52.7%)	26 (47.3%)	
Lymph node metastasis (n=26)			
Absent (n=9)	2 (22.2%)	7 (77.8%)	**0.014**
Present (n=17)	13 (76.5%)	4 (23.5%)	
Total	15 (57.7%)	11 (42.3%)	

2.4.6. Immunolabeling and comparative study of CRYAB in the mammary gland adjacent to neoplastic tissues

Mammary gland adjacent to benign neoplasms was present in 12 of the 15 samples (80.0%), being characterized by a staining intensity mostly moderate (n=6; 50.0%) while PS was mostly 1 to 25% (n=7; 58.3%).

Mammary gland adjacent to malignant neoplasms was present in 28 out of 55 samples (50.91%), with similar immunohistochemical findings: SI was mostly moderate (n=18; 64.3%) and PS was predominantly 1 to 25% (n=16; 57.1%) (Fig. 2e and 2f). So, when comparing CRYAB's expression of the adjacent mammary glands (AMG), either in benign or malignant neoplasms, we

found no significant differences in GS (p=0.484). In both benign and malignant groups, the staining pattern was usually heterogeneous. Results are shown in Table 9.

Table 9 - CRYAB immunolabeling of the adjacent mammary gland of benign and malignant tumors

CRYAB		AMG of Benign Neoplasms n (%)	AMG of Malignant Neoplasms n (%)
SI	Absent	1 (8.3%)	1 (3.6%)
	Weak	4 (33.3%)	7 (25.0%)
	Moderate	6 (50.0%)	18 (64.3%)
	Strong	1 (8.3%)	2 (7.1%)
PS	0%	1 (8.3%)	1 (3.6%)
	1-25%	7 (58.3%)	16 (57.1%)
	26-50%	2 (16.7%)	7 (25.0%)
	51-75%	2 (16.7%)	4 (14.3%)
	>75%	0 (0%)	0 (0%)
GS	Low	9 (75.0%)	17 (60.7%)
	High	3 (25.0%)	11 (39.3%)
	Total	12 (100%)	28 (100%)

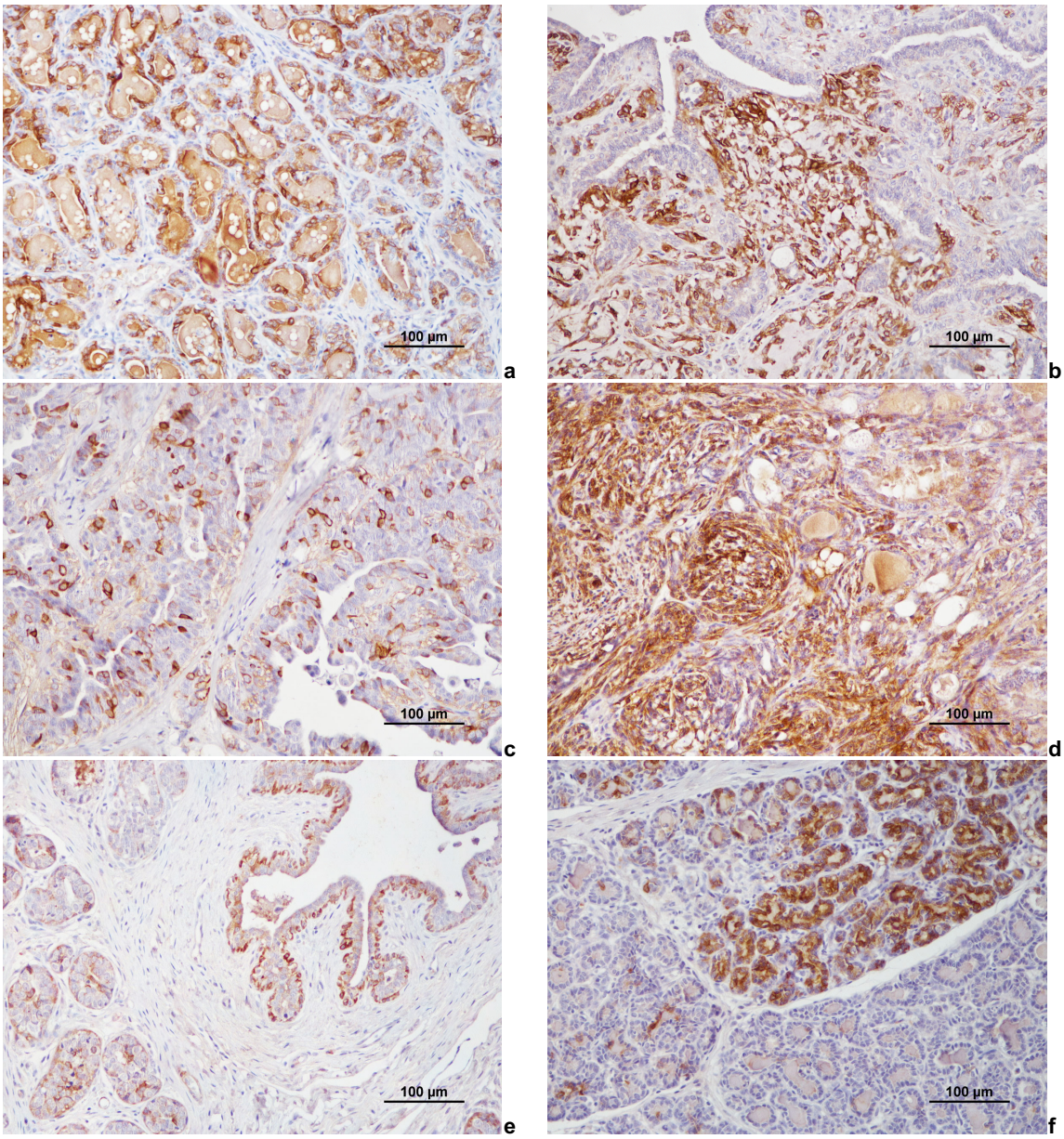

*Figure 2 - Immunohistochemical expression of CRYAB in canine mammary tissues: **a**, lobular hyperplasia showing epithelial positivity (strong intensity, in more than 50% of cells); **b**, complex adenoma, characterized by positivity of neoplastic myoepithelial cells (strong intensity, in more than 50% of cells); **c**, tubulopapillary carcinoma showing few positive neoplastic cells (moderate intensity, in less than 25% of cells); **d**, carcinoma and malignant myoepithelioma, with positive neoplastic cells (strong intensity, in more than 75% of cells); **e**, adjacent mammary gland, with moderate positivity of epithelial and myoepithelial cells in the lobules and positive myoepithelium in the duct; **f**, adjacent mammary gland, with an heterogeneous pattern of expression, with the presence of lobules with strong positivity (less than 50% of epithelial cells).*

2.5. Discussion

As stated before, canine mammary tumors are considered the most accurate animal model to study human breast cancer, a deadliest disease with specific therapeutic targets yet to be found. Female dogs usually share the same environment as women, meaning they probably are exposed to similar carcinogens. Besides that, canines and humans have common features such as body size and genetic variability, which make female dogs a better spontaneous animal model to study human breast cancer than other models such as laboratory animals (Sorenmo et al., 2011; Carvalho et al., 2014).

With research in CMT's moving at high rhythm due to the previously reported fact, molecular mechanisms underlying this type of cancer have been in the center of major investigations worldwide. As more and more of these mechanisms are being studied, more intervenient molecules and features are discovered, being one of them CRYAB. CRYAB has been studied since 1983 (Delaye & Tardieu, 1983), but few studies are found to be relevant or conclusive, and those that are, are often contradictory. We only found one research article regarding CRYAB in CMT's (Guvenc et al., 2012), which arouse our interest in this marker.

In the human normal breast tissue, CRYAB was found to be expressed by the myoepithelium and not expressed by the luminal epithelial component of the mammary epithelium (Koletsa et al., 2014). In our study, we found a weak and inconstant expression of CRYAB in the myoepithelium of normal/hyperplastic mammary gland samples, but also a heterogeneous pattern of expression in the luminal component, frequently with moderate/strong intensity, especially in hyperplastic tissues. The only study performed so far on CRYAB expression in canine mammary tissues described that only few luminal epithelial cells were positive in normal samples, with basal myoepithelial cells negative (Guvenc at al., 2012). These investigators also analysed two samples of hyperplasia that showed less than 25% of epithelial positivity. These discrepancies might be explained by the low number of samples analysed, but seem to indicate that in the canine species, CRYAB is not consistently expressed by myoepithelial cells of normal mammary gland. One hypothesis is that its expression might be associated with the estrous cycle stages, so, it would be interesting to study CRYAB expression in mammary gland tissues along the estrous cycle.

Koletsa et al. (2014) performed a large study on a series of almost 1000 human breast samples. They found that all the tissues with a high PS (>30%) presented a strong SI. They also found that malignant neoplasms with high histological grade (grade 3) were more frequently associated with high expression of CRYAB. Besides that, they also found a relation between CRYAB overexpression and the triple negative breast cancer samples. In our study, the neoplastic

myoepithelial cells were also overexpressing CRYAB when compared to the normal/hyperplastic tissues. It is known that canine mammary carcinomas are frequently characterized by a basal/myoepithelial phenotype (Gama et al., 2008), so a comparative study between CRYAB expression and the basal-like/triple-negative phenotype should be considered in this animal model.

In the present study, we found significant differences between histological groups. Benign tumors were predominantly characterized by high expression, especially due to the inclusion of histological subtypes, with myoepithelial cell proliferation (complex/mixed tumors), which showed increased levels of positivity.

Malignant tumors were positive in a small percentage (26/55; 47.3%), also showing CRYAB expression predominantly associated with non-simple carcinomas, with concomitant luminal cell and myoepithelial proliferation. Moreover, in malignant tumors, CRYAB expression was significantly associated with the histological subtype, even when considering the novel classification proposed by Goldschmidt et al. (2011).

Giving CRYAB's anti-apoptotic features (Kamradt et al., 2002; Malin et al., 2016) and its proved involvement in tumor growth (Kamradt et al., 2005), we expected to find an overexpression of this protein on larger tumors, which did not happen in our study. Instead, tumors with less than 3 cm represented 55.56% (n=20) of the tumors obtaining high scores, while only 27.78% (n=10) of these were larger than 5 cm; this lead to significant differences when comparing the GS and tumor size. The studies performed by Kamradt and its colleagues (2002) were conducted either in cell cultures or murine models of mammary cancer, which could explain the differences obtained. It would be interesting to study apoptotic and anti-apoptotic cell markers and compare their expression to CRYAB. In human breast cancer, Kim et al. (2015) showed that Bcl-2, an anti-apoptotic protein, was significantly associated with CRYAB, although a weak negative correlation was found.

Since CRYAB is connected to poor prognosis (Moyano et al., 2006; Koletsa et al., 2014), we expected that somehow it could be related to metastasis so that we could use it as a marker of dissemination. However, our results show that CRYAB expression was higher when metastases were absent. Previously reported studies stated that CRYAB overexpression is linked to lymph-node involvement, which goes in disagreement with our findings (Chelouche-Lev et al., 2004). Some authors have linked CRYAB overexpression with the presence of brain metastasis (Malin et al., 2014). Differences may be justified by the fact that, although the female dog is a potential animal model to study human breast cancer, differences do exist; one of them is the fact that

canine mammary carcinomas are not frequently characterized by the proliferation of myoepithelial cells (Sleeckx et al., 2011). Guvenc et al. (2012) also found a high positivity in complex carcinomas of the female dog, but a comparative study between histological subtypes was not performed in their study.

Adjacent mammary glands do not seem to be affected by tumor environment since we did not found significant differences between CRYAB's expression in adjacent mammary glands to benign or malignant tumors (p=0.484). In our review of literature, we did not found any supportive theories that CRYAB could interfere with the tumor environment and further promote neighbor cells modifications.

Our study, although meticulously performed, has a small number of samples, which may explain some of the differences found with the reviewed studies. However, the only report of CRYAB in CMT's was performed on an even smaller series than ours (Guvenc et al., 2012), which lead us to believe that our findings regarding CMT's may be more accurate. In addition, there were different methodologies adopted to quantify CRYAB expression, which can also explain the differences observed. We would also like to emphasize that the lack of some clinicopathological information may falsely influence the results shown, since that information mostly relies on reports from our colleagues, which are usually incomplete.

In the future, CRYAB expression should be evaluated in prognostic studies, so that we can accomplish if it is correlated with prognosis. In human breast cancer, a link to poor prognosis was found, but not when considering CRYAB as an individual factor of prognosis (Koletsa et al., 2014). We could also compare CRYAB expression with other markers like ER, PR and AR, so that we can conclude if it is linked to tumors with triple negativity in CMT's as it is in human breast cancer (Koletsa et al., 2014).

3. L1CAM

3.1. Review

3.1.1. Structure

The L1 cell adhesion molecule (L1CAM) is a transmembrane glycoprotein composed by a highly conserved cytoplasmic tail, a transmembrane region, five fibronectin type III repeats and six immunoglobulin-like domains, with an weight of approximately 200 to 220 kDa (Kiefel et al., 2012; Samatov et al., 2016). Its existence was first reported by Rathjen and its colleagues in 1984 as a cell surface antigen in the central nervous system (cerebellar cortex of mice) (Rathjen & Schachner, 1984).

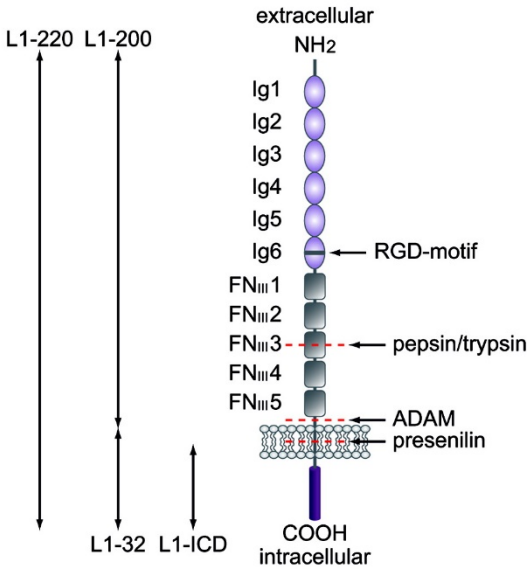

Figure 3 – L1 CAM structure and cleavage sites (adapted from Kiefel et al., 2012)

The cell adhesion molecules (CAM's) are responsible for cell-cell interaction, as well as cell-extracellular matrix (ECM) interaction. The CAM's are composed by five types of molecules: cadherins, integrins, selectins, mucins and immunoglobulin superfamily proteins, being the later the group of proteins in which L1CAM is included (Homrich et al., 2015; Samatov et al., 2016).

3.1.2. Expression

Expression of L1CAM is regulated by a variety of factors, which include the RE1-Silencing Transcription factor (REST) and the nuclear factor 1-A (NF-1A) as suppressors and the transcription factor Slug/SNAI2 and T-cell factor activated by treatment with TGF-β1 and β -catenin signaling as promoters (reviewed by Kiefel et al., 2012; Samatov et al., 2016).

It is expressed by neural, hematopoietic and transformed epithelial cells (H. Schäfer & Müerköster, 2009).

3.1.3. Interactions/Function

L1CAM was originally described as a cell surface glycoprotein in the mice brain (Rathjen & Schachner, 1984), which was responsible for neuron survival and neurite outgrowth, as a normal intervenient in the development of the nervous system (Chen et al., 1999; Bao et al., 2008).

It is now known that this molecule has other functions and can establish a variety of interactions. These interactions can occur in a homophilic (interacting with itself) or heterophilic way (with other types of molecules)(Homrich et al., 2015). These interactions can still be classified as *cis* or *trans* interactions either if they occur between the same membrane cell or between adjacent cells, respectively.

Each part of the complex glycoprotein is responsible for a different type of interaction. For example, the Ig-like domains are responsible for either homophilic (Ig1-4) (Gouveia et al., 2008) and heterophilic interactions as well. In the heterophilic way, it can bind to other components of the Ig superfamily (such as N-CAM or TAG-1/axonin), to the CD 24 glycoprotein or to the neuropilin-1 molecule (reviewed by Samatov et al., 2016). There is also the possibility to interact with the group of receptor tyrosine kinases like the EGFR, to which the Ig domains of L1CAM bind during the normal development of the nervous system (Donier et al., 2012). One of the most important heterophilic interactions of L1CAM is the ability to bind to integrins (Mechtersheimer et al., 2001). The RGD-motif (located in the Ig-6) is responsible for this connection, promoting further activation of focal adhesion kinase (FAK), which changes its conformation to bind to Src. This FAK/Src complex then activates signaling pathways such as mitogen-activated protein kinases (MAPK) (reviewed by Samatov et al., 2016).

On the other hand, the fibronectin repeats are responsible for the modulation of the neuronal differentiation and have a role on the interaction with integrins. The intracellular part is mostly responsible for the interaction of L1CAM with the cytoskeleton, trough binding with ankyrins

and regulating gene expression in the nucleus (Gast et al., 2008; reviewed by Herron et al., 2009; Kiefel et al., 2012; Samatov et al., 2016).

L1CAM is also believed to induce the nuclear factor κB (NF-κB) through an increased production of interleukin 1β. (Kiefel et al., 2010, 2012). Kiefel and her colleagues proved that L1CAM not only activates the MAPK pathway but is also involved in the activation of the NF-κB. Through induction of EMT with TGF- β, they observed that there is an increased expression of L1CAM in breast cancer and pancreatic ductal adenocarcinoma cell lines, besides the fact that it is overexpressed in endometrial carcinoma too, which they had proven before.

In order to facilitate the communication and further research regarding L1CAM, the interactions of the molecule were classified in 3 different types (reviewed by Kiefel et al., 2010):

The well know MAPK pathway, which is vulgarly known as L1CAM "assisted" signaling, consisting of the interaction of the molecule with cell membrane surface components (neuropilin, EGFR, FGFR) and consequent activation of the MAPK pathway.

The pathway including the proteolytic cleavage of L1CAM (ectodomain shedding), which is called L1CAM forward signaling. This proximal cleavage (carried by ADAM 10 or 17) is responsible for the emergence of a soluble extracellular domain (ectodomain) and an intracellular domain (ICD). The first is responsible for the interaction with integrins, since the RGD-motif is still intact, allowing it to still act as a substract. Consequently, this ectodomain can still activate the NF-κB through the activation of ERKs. The ICD is then processed by a gamma secretase, giving rise to a smaller segment that is capable of translocating to the nucleus where it can regulate the transcription and consequent expression of genes. This translocation to the nucleus is still not fully understood.

The last pathway described for L1CAM is known as L1CAM reverse signaling and consists in the interaction of a full, not cleaved molecule. L1CAM is, once again, capable of interacting with integrins (RGD-motif), activating the NFκB, as described before.

3.1.4. L1CAM in Cancer

Regarding human cancer, L1CAM has been reported in a variety of tumors including ovarian, endometrial, breast, gastric, gallbladder, hepatocellular, renal cell, prostate and other carcinomas. It has also been identified in melanoma, glioma, neuroblastoma, thyroid anaplastic carcinoma, pancreatic ductal adenocarcinoma, among others (reviewed by Altevogt et al., 2016).

Its role has been associated with chemotherapy resistance, increased cell motility, high malignancy, high histological grades, advanced tumor stages, as well as with the epithelial to

mesenchymal transition (EMT) (Bao et al., 2008; Gast et al., 2008; Li & Galileo, 2010; Kiefel et al., 2012; Yi et al., 2014; Altevogt et al., 2016; Samatov et al., 2016).

L1CAM has been suggested to have an important role in EMT (Shtutman et al., 2006; Kiefel et al., 2012), which is an important step in the malignancy of tumors, representing the loss of the cell-to-cell adherence (Hanahan & Weinberg, 2000). This loss of adherence, together with the loss of the apical-basal polarity represents an important step in allowing tumor cells migration and consequent metastasis formation (Christiansen & Rajasekaran, 2006; Polyak & Weinberg, 2009). This mimics the normal process of grastrulation in which the epithelial sheet of the ectoderm forms a third germinal layer called mesoderm (Shtutman et al., 2006). Migratory cells of this last layer represent mesenchymal cells. The role of L1CAM in EMT is still not fully understood but we now know that it is related to the disruption of the E-Cadherin-mediated adherens and to an increased β-catenin dependent transcription, resulting in a phenotype with increased motility (Shtutman et al., 2006). This phenotypic change can be confirmed with immunohistochemical profiles in which we expect the cells to lose epithelial markers (such as E-cadherin and cytokeratins) and express mesenchymal markers, like vimentin (Kiefel et al., 2012; Peña et al., 2014; Samatov et al., 2016).

As far as we know, L1CAM was never reported in canine mammary tumors.

More recently, L1CAM has been correlated to angiogenesis in a variety of human types of cancer. It has been suggested that the overexpression of the molecule in cancer cells promotes adhesion to endothelial cells (ECs), promoting their progression, migration and further angiogenesis (Dippel et al., 2013; Magrini et al., 2014, 2015; Burgett et al., 2016), even though it is not expressed by normal vasculature, or is expressed at a low level (Issa et al., 2009; Maddaluno et al., 2009; Magrini et al., 2014). Magrini et al. proved that the endothelial deficiency of L1CAM resulted in a reduced tumor angiogenesis and promoted the normalization of the vessels, since newly formed tumor vessels are heterogeneous, tortuous and have an uneven lumen. Endothelial L1CAM is overexpressed when stimulated by a variety of factors such as TNF-α, INF-ɣ, TGF-β1 and VEGF-A, which are classical angiogenic factors (Issa et al., 2009). It has also been connected to the transendothelial migration of dendritic cells, leaving sight to suppose that it can also promote transendothelial migration of other cell types (Maddaluno et al., 2009). In glioblastomas, the heterophilic interaction between L1CAM in cancer cells and integrins in endothelial cells promoted a phenotypical change of the latter to a more angiogenic phenotype, thus augmenting the migration potential of the ECs, therefore promoting angiogenesis (Burgett et al., 2016). Heterophilic binding was also observed in breast cancer cells, binding L1CAM to ALCAM and to

E-selectin (Dippel et al., 2013). However, homophilic binding was also found on this latter project, where it was found to be dependent of TNF-α stimulation of ECs.

3.1.5. L1 CAM as a marker of prognosis

In terms of prognosis L1CAM is related to poor clinical outcome/prognosis, since it is responsible for at least part of the phenotypical changes that result in tumor aggressiveness. Pediatric neuroblastoma is the only exception in which L1CAM overexpression is related to good prognosis, although the reasons why this happens are yet to be discovered. Besides that, and as stated before, L1CAM overexpression is related to higher risk of metastasis, higher grades, chemotherapy resistance, EMT, and others (M. K. E. Schäfer & Altevogt, 2010; Kiefel et al., 2012; Zhang et al., 2015; Samatov et al., 2016).

3.2. Objectives

The goal of this study was to report, for the first time, the expression of L1CAM in canine mammary cancer. From the files in our power, we decided to investigate if there were any differences between inflammatory and non-inflammatory mammary cancer. Further research was made regarding our findings in tumor vessels and previously studied angiogenic factors on the same samples. Although not the object of study of the current dissertation, a small approach will be made.

3.3. Material and Methods

3.3.1. Tumor specimens

The specimens were obtained from the files of the Department of Animal Medicine, Surgery and Pathology of the Complutense University of Madrid. From the available archival material, we selected 27 malignant neoplasms, composed by canine inflammatory mammary cancer (n=12; 44.4%) and non-inflammatory canine mammary cancer (n=15; 55.6%). The material had been fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections were cut and stained with hematoxylin and eosin (HE) for histological examination.

3.3.2. Histopathological parameters evaluation

All tumor samples were revised from HE stained sections, according to the new classification proposed (Goldschmidt et al., 2011). The goal of our study was to determinate whether there were differences or not between inflammatory mammary cancer (IMC) and non-inflammatory mammary cancer (NIMC), and not between different histological diagnosis. Other histopathological parameters evaluated included: lymphovascular invasion (presence vs. absence).

Histological grade was evaluated as for CRYAB, being classified from 1 to 3. All tumors of the present study were classified as grade 3 tumors (high grade).

Clinical parameters were not included in this study.

Other parameters had been previously studied on the same samples, including E-cadherin, factor VIII, CD34, VEGFA, Cox-2, VEGF-D, VEGFR-3, hormonal receptors (ERα, AR, PR) and metastasis in various organs (lungs, lymph nodes, kidney, bones, heart, spleen, muscle, liver, pancreas, among others). However, this data will not be presented on the present master thesis since it was not the goal of the study performed with Prof. Laura Peña during my externship at the UCM.

3.3.3. Immunohistochemistry

Immunohistochemistry was performed by our colleagues in the United States, under the supervision of Dr. Hugo Arias-Pulido at the New Mexico Cancer Center.

3.3.4. Quantification of immunolabeling

Quantification of immunolabeling in these specimens was executed by two observers (FM and LP) according to the Allred score (Allred et al., 1998), where the Total Score (TS) was obtained

by adding the Stain intensity (SI) to the Percentage of Staining (PS). Tumor cells SI was classified as absent (0), weak (1), moderate (2) or strong (3) and PS was classified as no labeling (0), <1% (1), 1-10% (2), 10-33% (3), 33-66% (4) and >66% (5) giving a TS of 0 or 2 to 8. These characteristics are summarized on the following table.

Table 10 – Quantification of L1CAM immunolabeling (adapted from Allred et al., 1998)

Stain Intensity (SI)	Percentage of Staining (PS)	Total Score (TS=SI+PS)
0 (Absent) 1 (Weak) 2 (Moderate) 3 (Strong)	0 (0%) 1 (<1%) 2 (1-10%) 3 (10-33%) 4 (33-66%) 5 (>66%)	0 or 2 to 8

The staining pattern was also classified as cytoplasmic, membranous or both. Membranous staining was further divided into continuous or discontinuous staining but we turned out to discover that all the samples presented the discontinuous pattern, when membranous staining was present, so we won't present this division on the results.

As we progressed in our study, we noticed a pattern in vessels staining so we also classified them regarding the positivity of endothelial cells. Vessels within the tumor, vessels on the normal area of the sample and vessels containing emboli were individually classified as negative (when all endothelial cells were negative to L1CAM), positive (when all endothelial cell from all the vessels found were positive to L1CAM) and irregular when some of them were positive and others were negative, leading to incoherence in endothelial cells staining.

3.3.5. Statistical methods

All variables except for L1CAM total score (continuous) were treated as categorical variables. Therefore, Mann-Whitney test was applied to L1CAM total score and Chi-Square test was applied to the rest of the variables. p value <0.05 was considered significant. IBM SPSS Statistics 22 and 24 softwares were used.

3.4. Results

3.4.1. Immunolabeling of L1CAM in malignant neoplasms

Most of the tumors demonstrated a strong stain intensity (n=12; 44.4%) as well as more than 66% of the epithelial cells stained (n=11; 40.7%) (Fig. 4a). This lead to a majority of the tumors obtaining the top total score possible, with 33.3% (n=9) of tumors obtaining a total score of 8 points. Cytoplasmic staining was found in 24 out of 27 (88.9%), but only in 10 out of 24 (41.7%) if we refer to it alone (without a concurrent membranous staining). Although we did find membranous staining we could only find it in association with the cytoplasmic pattern, meaning that the exclusively membranous pattern was never present. Membranous and cytoplasmic pattern together was found in 14 out of 24 cases (58.3%). Regarding the endothelial cells (ECs), most of the ECs from the tumor environment as well as the ones from vessels with emboli were negative to L1CAM expression (n=14; 56% and n=7; 53.8%, respectively) but vessels from normal areas of the mammary gland were mostly positive (n=9; 69.2%) (Fig. 4d, e and f).

Table 11 - Immunolabeling characteristics of L1 CAM in epithelial and endothelial cells

L1 CAM	n (%)
Stain Intensity (SI) of *Epithelial Cells* (n=27)	
Absent	3 (11.1%)
Weak	2 (7.4%)
Moderate	10 (37.0%)
Strong	12 (44.4%)
Total	27 (100%)
Percentage of Staining (PS) of *Epithelial Cells* (n=27)	
0%	3 (11.1%)
<1%	2 (7.4%)
1-10%	5 (18.5%)
10-33%	2 (7.4%)
33-66%	4 (14.8%)
>66%	11 (40.7%)
Total	27 (100%)
Total Score (TS= SI+PS) of *Epithelial Cells* (n=27)	
0	3 (11.1%)
2	0 (0%)
3	3 (11.1%)
4	4 (14.8%)
5	1 (3.7%)
6	4 (14.8%)
7	3 (11.1%)
8	9 (33.3%)
Total	27 (100%)
Staining Pattern of *Epithelial Cells* (n=24)	
Exclusively Membranous	0 (0%)
Membranous and Cytoplasmic	14 (58.3%)
Exclusively Cytoplasmic	10 (41.7%)
Total	24 (100%)

Table 11 - Immunolabeling characteristics of L1 CAM in epithelial and endothelial cells (cont.)

L1 CAM	n (%)
Staining of *Endothelial Cells of Tumor Vessels* (n=25)	
Negative	14 (56.0%)
Irregular	10 (40.0%)
Positive	1 (4.0%)
Total	25 (100%)
Staining of *Endothelial Cells of normal area* of the mammary gland vessels (n=13)	
Negative	2 (15.4%)
Irregular	2 (15.4%)
Positive	9 (69.2%)
Total	13 (100%)
Staining of *Endothelial Cells of vessels with emboli* (n=13)	
Negative	7 (53.8%)
Irregular	4 (30.8%)
Positive	2 (15.4%)
Total	13 (100%)

3.4.2. Comparative study of L1CAM expression between the inflammatory and non-inflammatory mammary cancer groups

Regarding the SI there was no significant differences found in our samples (p=0.414). The PS was significantly different between the two groups (p=0.009) with 75% (n=9) of the IMCs having more than 66% of their epithelial cells stained (Fig. 4a) while only 13.3% (n=2) of the NIMC did. L1CAM total score was also found to be significantly different between the two groups with p=0.003. The inflammatory group was found to have an average TS of 7.08 ± 1.505 (Fig. 4a) *versus* the non-inflammatory which had an average TS of 4.13 ± 2.669 (Fig. 4b and c). The staining pattern was also significantly different, with the inflammatory group presenting mostly a cytoplasmic and membranous pattern, while the non-inflammatory group presented mostly the exclusively cytoplasmic pattern (p=0.036). Results are presented on table 12.

Table 12 - Comparative study of L1CAM expression in epithelial cells with the inflammatory and non-inflammatory mammary cancer groups

L1CAM	Non-Inflammatory mammary cancer	Inflammatory mammary cancer	*p* Value
Staining Intensity (SI) (n=27)			
Absent	3 (20%)	0 (0%)	
Weak	1 (6.7%)	1 (8.3%)	
Moderate	6 (40%)	4 (33.3%)	0.414
Strong	5 (33.3%)	7 (58.3%)	
Total	15 (100%)	12 (100%)	
Percentage of Stain (PS) (n=27)			
0%	3 (20%)	0 (0%)	
<1%	2 (13.3%)	0 (0%)	
1-10%	4 (26.7%)	1 (8.3%)	
10-33%	2 (13.3%)	0 (0%)	**0.009**
33-66%	2 (13.3%)	2 (16.7%)	
>66%	2 (13.3%)	9 (75.0%)	
Total	15 (100%)	12 (100%)	
Total Score (TS) (n=27)			
	15 (100%) Medium= 4.13 ± 2.669	12 (100%) Medium= 7.08 ± 1.505	**0.003**
Staining Pattern (n=24)			
Exclusively Membranous	0 (0%)	0 (0%)	
Membranous and Cytoplasmic	4 (33.3%)	10 (83.3%)	**0.036**
Exclusively Cytoplasmic	8 (66.7%)	2 (16.7%)	
Total	12 (100%)	12 (100%)	

3.4.3. Comparative study of L1CAM expression in ECs of the inflammatory and non-inflammatory mammary cancer groups

When comparing the staining of endothelial cells between the two groups, no significant differences were found. However, tumor vasculature tended to show less expression of the marker than the normal vasculature, with 56% of tumor samples being negative to the marker (14 out of 25).

Table 13 - Comparative study of L1CAM expression in endothelial cells of the inflammatory and non-inflammatory mammary cancer groups

L1CAM	Non-Inflammatory mammary cancer	Inflammatory mammary cancer	*p* Value
ECs in Tumor Vessels (n=25)			
Negative	8 (57.1%)	6 (54.5%)	0.0669
Irregular	6 (42.9%)	4 (36.4%)	
Positive	0 (0%)	1 (9.1%)	
Total	14 (100%)	11 (100%)	
ECs in normal area of the mammary gland (n=13)			
Negative	1 (16.7%)	1 (14.3%)	1.0
Irregular	1 (16.7%)	1 (14.3%)	
Positive	4 (66.7%)	5 (71.4%)	
Total	6 (100%)	7 (100%)	
ECs of vessels with emboli (n=13)			
Negative	3 (75.0%)	4 (44.4%)	0.276
Irregular	0 (0%)	4 (44.4%)	
Positive	1 (25.0%)	1 (11.1%)	
Total	4 (100%)	9 (100%)	

3.4.4. Comparative study of L1CAM TS with the expression of L1CAM by ECs in tumor and embolized vessels

The following non-parametric correlations were obtained using Kendall's tau_b test. A correlation coefficient of 0.150 an 0.400 was found between L1CAM TS with ECs of tumor vessels and ECs of embolized vessels, respectively, meaning that these variables are positively

associated, but not on a significant level, since *p* values were higher than 0.05, as presented on the next table.

Table 14 - Comparative study of L1CAM TS with the expression of L1CAM by ECs in tumor and embolized vessels

	ECs tumor vessels	ECs embolized vessels
L1 CAM Total Score	*p*=0.392	*p*=0.117

3.4.5. Other comparative studies

Other comparative studies were performed, comparing L1CAM expression with the expression of multiple other markers that Prof. Laura Peña had previously studied on the same samples. These included: E-cadherin, factor VIII, CD 34, COX-2, VEGFA, VEGF-D, VEGFR-3, AR, ER (α and β) and PR. It was also compared with the presence/absence of metastasis in multiple organs, from the most typical ones, as the lungs and lymph nodes, to others such as the ovarium, kidneys, bones, brain and others. It was also compared with ki67 index. This study was not an integral part of my internship in the UCM with Prof. Laura Peña, so that we chose not to include it on the present dissertation. Just as a curiosity we would like to reveal that we did find that the expression of L1CAM by endothelial cells of inflammatory mammary cancer vessels containing emboli showed an inverse association with factor VIII (*p*=0.015) and VEGFR3 (*p*=0.050) expression.

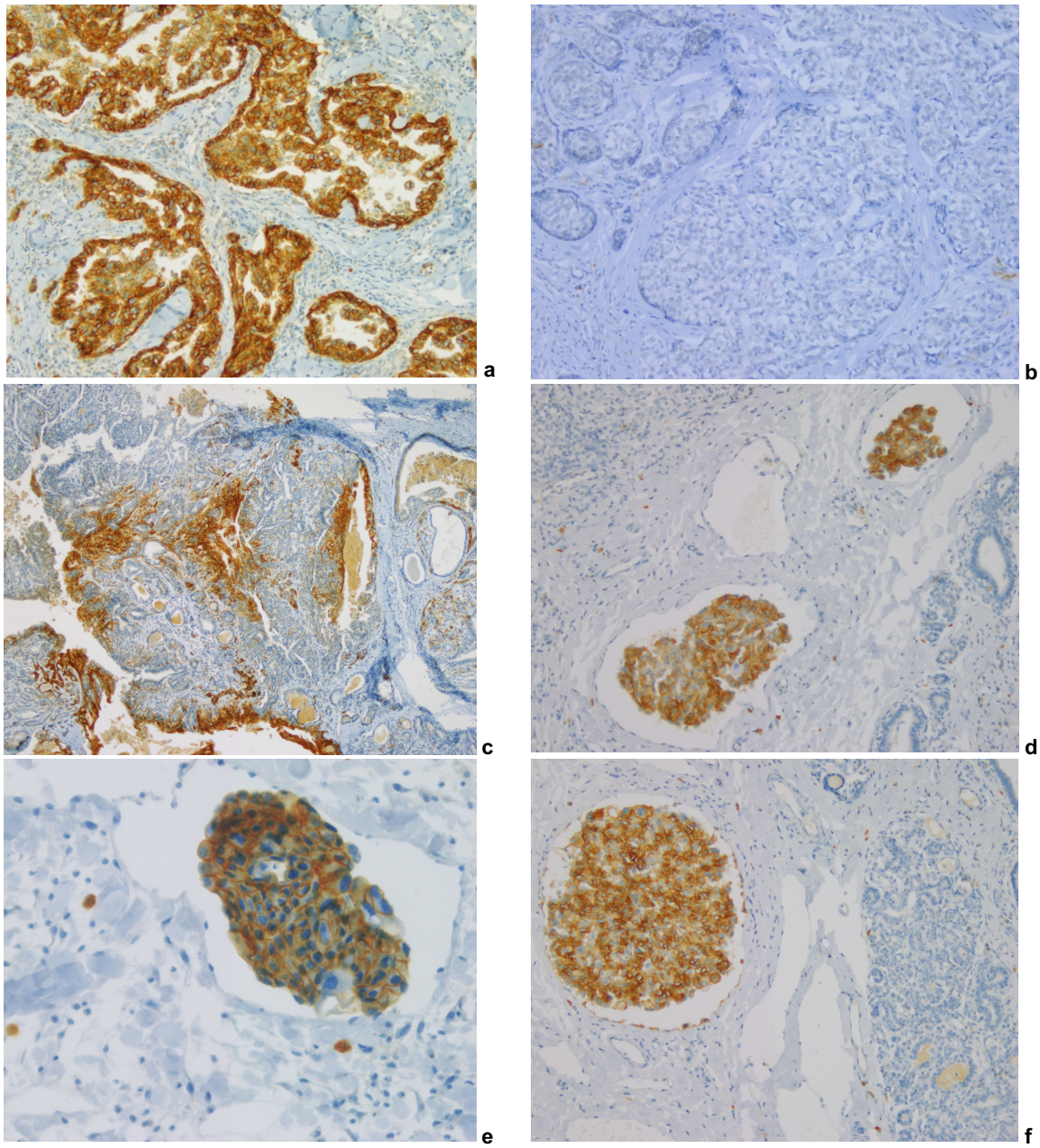

Figure 4 - Immunohistochemical expression of L1CAM in canine mammary tissues: ***a,*** *inflammatory carcinoma showing epithelial positivity (strong intensity in more than 66%of cells) (10x);* ***b,*** *non inflammatory carcinoma characterized by negativity of neoplastic cells (10x);* ***c****, non inflammatory carcinoma showing moderate to strong epithelial positivity in less than 10% of cells (4x);* ***d,*** *inflammatory carcinoma with two positive emboli within negative vessels (10x);* ***e,*** *inflammatory carcinoma characterized by the presence of a positive embolous within a negative vessel (20x);* ***f,*** *inflammatory carcinoma characterized by the presence of a positive embolous within a positive vessel (20x).*

3.5. Discussion

Inflammatory mammary cancer (IMC) is the most aggressive form of mammary cancer (female dogs) and breast cancer (women), being its main histologic characteristic the invasion of lymphatic vessels of the dermis. It is considered a different type of breast/mammary cancer, with different carcinogenic mechanisms, and involving increased angiogenesis and high vascular invasiveness and embolization (Pérez Alenza et al., 2001; Giordano & Hortobagyi, 2003; Peña et al., 2003; Van der Auwera et al., 2004).

L1CAM was found to be overexpressed in inflammatory mammary cancer when compared to the non-inflammatory group. The molecule had a medium total score of 7.08 in IMC while only 4.13 of TS was obtained regarding the NIMC. *p* value was of 0.003, meaning that differences were statistically significant.

Giving all the similarities described before in the introduction, female dogs are currently the best animal model to study inflammatory breast cancer that, despite being rare, is the most aggressive and deadliest form of breast cancer. Our findings go in agreement with these statements, since L1CAM is usually associated with poor clinical outcome, metastasis formation, angiogenesis, augmented cell motility, among other features that lead to tumor aggressiveness and malignancy (Bao et al., 2008; Gast et al., 2008; Li & Galileo, 2010; Kiefel et al., 2012; Yi et al., 2014; Altevogt et al., 2016; Samatov et al., 2016). High TS scores of L1CAM in IMC samples reveal that the inflammatory phenotype is more aggressive than the NIMC one. The differences were also significant regarding the PS, with p=0.009. Nine of the IMCs (75%) had more than 66% of the cells stained, while the NIMC group tend to have between 1 and 10% of them (n=4; 26.7%).

The staining pattern was also found to be significantly different (p=0.036), with 10 out of 12 (83.3%) of the IMC presenting a membranous and cytoplasmic staining, while most of the NIMC group showed an exclusively cytoplasmic staining. Besides our efforts to understand these differences, we did not found any information in the literature regarding the staining pattern and the malignancy or the inflammatory group. We do know that L1CAM can be submitted to an ectodomain cleavage, which we hypothesize could make the marker loose the membranous stain (Kiefel et al., 2010). However, this ectodomain shedding (described in 2.1.3) usually represents the majority of the interactions of L1CAM, meaning it should be associated with worse prognosis. In our case, it is related to NIMC and not to IMC, meaning it is related to better prognosis. Although, we do want to emphasize that this is a mere hypothesis and that we did not found any association between the stain pattern and the interactions of L1CAM in the literature revised.

Regarding the vessels, we did not found any significant differences regarding L1CAM expression by ECs in IMC or NIMC. We expected to find some differences since L1CAM is usually associated with angiogenesis and metastasis formation. Its overexpression is usually related to a higher adherence of tumor cells to EC, which will make them progress, migrate and potentiate their angiogenic features (Dippel et al., 2013; Magrini et al., 2014, 2015; Burgett et al., 2016). With that being said, we expected to find an overexpression of L1CAM in the inflammatory group, since we know this group is characterized by an increased angiogenesis and high vascular invasiveness and embolization, as previously stated in this point. Of note that normal vessels expressed more L1CAM than the rest, going against the statements that normal vessels don't express L1CAM (or express it at low levels) and that it is overexpressed in pathologic situations, such as inflammation or tumors (Issa et al., 2008; Maddaluno et al., 2009; Magrini et al., 2014).

With that in mind, we then decided to compare L1CAM results with some previously studied markers on the same samples. Two of these markers, VEGFR3 and factor VIII, both lymphangiogenic markers, showed an inverse association with L1CAM expressed by ECs of vessels containing emboli (p= 0.050 and 0.015, respectively). VEGF-D showed a tendency to a positive association with L1CAM overexpression, although not significative. This can lead to assume that L1CAM is in fact related to angiogenesis and, consequently, tumor aggressiveness, metastasis formation and poor prognosis.

Further studies should be performed in CMT's to test if L1CAM is in fact related to EMT, by testing different basal or luminal markers, as recommend in published guidelines (Peña et al., 2014) and comparing its expression with L1CAM overexpression. Interesting studies in overall survival time could also be performed. Studies in breast cancer would also be interesting to compare L1CAM expression in inflammatory and non-inflammatory breast cancer, to accomplish if there is any relationship as we showed there is in canines.

4. CONCLUSIONS

By the end of this study, I am able to conclude that research in breast/mammary cancer is way more complex than I originally thought. A wide variety of molecules are currently on the focus of a variety of researchers around the globe. From newly discovered molecules, to older ones, breast/mammary cancer is a complex entity with multiple features yet to be discovered.

In our study, we were able to work with two of those molecules: CRYAB and L1CAM. Our findings regarding both of the molecules reveal that both are promising future markers for breast/mammary cancer.

Regarding CRYAB, our findings go mostly in disagreement with the only previously reported research in CMT's. It may be an interesting marker of myoepithelial proliferation either in canines or humans. In fact, significant differences were observed in CRYAB expression between different malignant histological subtypes, being predominantly expressed in non-simple carcinomas. Further studies are required to confirm our results, analyzing larger series that could promote better and more trustable results.

On the other side, L1CAM was in fact overexpressed in the inflammatory group, leading us to believe it is a promising marker of prognosis and may be, in the future, a possible therapeutic target in canine inflammatory mammary tumors. Further studies are required in women to accomplish if the differences we found in CMT's also happen in women. L1CAM may also be a marker of inflammatory breast cancer. Some authors already believe it is a marker for basal-like, triple negative breast tumors.

As a soon to be Doctor of Veterinary Medicine, I am proud to set forth these results and hopefully inspire other young researchers to work with breast/mammary cancer and help us progress in our knowledge about this deadliest disease. I really hope that these work and respective results can help someone, somewhere, evolve its knowledge in this field and provide information for further research and evolution.

This dissertation really improved my skills regarding immunohistochemistry, veterinary pathology and statistical methods. Besides all of my veterinarian skills, knowledge in human breast cancer was also acquired as part of my review of either L1CAM and αB-Crystallin, mostly studied in humans.

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