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TITOLO TESI

PHENOTYPIC CHARACTERIZATION OF THE EPITHELIAL AND MYOEPITHELIAL COMPONENTS IN CANINE AND FELINE MAMMARY TUMOURS

Presentata da: **Dott.ssa GERMANA BEHA**

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Esame finale anno 2014

Verbale della seduta del Collegio dei Docenti del Corso di Dottorato in Scienze Veterinarie del 11 Febbraio 2014

L'anno 2014, addì 11 del mese di Febbraio in Ozzano (BO), nell'Aula Gherardini del Dipartimento di Scienze Mediche Veterinarie, alle ore 14,00 si è riunito il Collegio dei Docenti del corso di Dottorato di Ricerca in Scienze Veterinarie.

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Il Coordinatore riconosce valida la seduta e la dichiara aperta per trattare, come dall'avviso di convocazione, il sottoindicato ordine del giorno:

1) Comunicazioni

2) Presentazione tesi Dottorandi

3) Richiesta Doctor Europaeus

4) Varie ed Eventuali.

1) Comunicazioni

Il Coordinatore comunica che in data 7 Febbraio ha partecipato ad una riunione indetta dal Prorettore alla Ricerca, Prof. Braga, allo scopo di definire i criteri di riparto delle risorse del dottorato, di provvedere all'attivazione del 30° ciclo e di discutere sulle linee guida ANVUR per l'accreditamento dei dottorati. Le informazioni più importanti riguardano:

- Il budget per l'attivazione del 30° Ciclo verrà assegnato direttamente al Dipartimento e sarà vincolato (possibilità di utilizzo solo per Borse di Dottorato); eventualmente, si potrà, invece, effettuare un "travaso" di risorse dal budget per assegni di ricerca e per Borse Marco Polo al budget per borse di Dottorato (ma non viceversa);
- Allo stato attuale, il budget assegnato al Dottorato in Scienze Veterinarie per il 30° Ciclo è superiore a quello assegnato il Ciclo precedente (il budget è stato definito sia in base alle performances del Dipartimento che di quelle del Dottorato; rispetto all'anno precedente, non poteva essere aumentato di più del 4%, non poteva essere ridotto di più del 10%. Il nostro Dottorato ha ottenuto il massimo); dovrebbe permettere l'attivazione di più di 7 borse (52,000 Euro/borsa) alle quali si dovrebbe sommare un residuo derivante da una cessazione (Dott.ssa Claudia Costanza);
- Il bando per il 30° Ciclo uscirà verso la metà di Aprile 2014; le selezioni devono essere ultimate entro il 30 Settembre. Il Dottorato dovrà iniziare o il 1 Ottobre o il 1 Novembre 2014;
- Il Collegio deve essere costituito solo da 16 membri (dei quali massimo 4 ricercatori); altri Docenti (fino ad un massimo complessivo di 32) possono partecipare ad un Collegio "allargato" ma non sono vincolanti (sono comunque tenuti ad avere un punteggio minimo di 2.2; vedi punto successivo);
- Il punteggio di ogni Docente partecipante al Collegio deve essere minimo di 2.2 (su un massimo di 3), secondo la valutazione VQR. Siccome tale punteggio, per motivi di privacy, è noto solo all'interessato, è opportuno (necessario) che tutti coloro che hanno un punteggio inferiore si dimettano dal Collegio e vengano sostituiti da Colleghi maggiormente qualificati;
- Il Tutor (supervisore) del Dottorando può essere un Docente (o ricercatore) esterno al Collegio, ma a sua volta deve avere un punteggio minimo di 2.2

Il Coordinatore comunica ancora di aver dato l'adesione del Dottorato di Scienze Veterinarie al programma "Ciencia sem Fronteiras" (CSF) promosso dal Governo Brasiliano e volto a concedere l'ammissione al Dottorato di giovani laureati brasiliani che potranno usufruire di borse finanziate interamente da quel Governo. Il Coordinatore invita pertanto tutti i componenti il Collegio a dare la loro disponibilità ad accettare eventuali domande di Candidati che si propongano di svolgere un'attività di ricerca in ambiti che sono di interesse degli stessi Docenti.

2) Presentazione tesi Dottorandi

Il Coordinatore fa presente al Collegio dei Docenti che i dottorandi, iscritti all'ultimo anno di corso, hanno provveduto a presentare, nei termini previsti, le dissertazioni finali.

Il Collegio è chiamato a redigere, per ciascuno di essi, la "presentazione" da allegare alla tesi finale.

Si invitano, a tal fine, i componenti del Collegio e/o i supervisori, che prevalentemente hanno guidato le attività di ricerca dei dottorandi a voler illustrare i contenuti delle predette tesi e i risultati conseguiti.

Dopo ampia discussione, sentiti anche i dottorandi in merito alle ricerche svolte, oggetto della dissertazione scritta, il Collegio dei Docenti decide unanime di approvare le **"presentazioni"** di seguito riportate le quali illustrano l'attività scientifico-formativa svolta durante il corso, mettendone in luce gli aspetti positivi o, eventualmente, negativi.

Germana Beha, Tutor Prof.ssa Cinzia Benazzi

Curriculum seguito: Sanità Animale

Titolo della tesi: **Phenotypic characterization of the epithelial and myoepithelial components in canine and feline mammary tumour.**

La dott.ssa Germana Beha nei tre anni di dottorato ha svolto ed approfondito in modo più che positivo gli argomenti che le sono stati affidati e che l'hanno portata ad ottenere sei pubblicazioni su rivista internazionale (in gran parte a primo nome) e 10 comunicazioni a convegni nazionali ed internazionali. La Dott. Beha ha dimostrato indubbie capacità di ideare e portare avanti i lavori in cui è stata coinvolta, oltre che costanza a perseguirne i risultati. Pertanto viene da tutti auspicato per la dott.ssa Beha il raggiungimento del titolo di Dottore di Ricerca; il giudizio finale è più che positivo.

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1. INTRODUCTION

Breast cancer represents a heterogeneous group of tumours with varied morphologic and biological features, behavior, and response to therapy (Rakha and Ellis, 2011). The above mentioned heterogeneity is well recognized and raises the need for a revision of the World Health Organization (WHO) classification, which was based on morphological features alone (Eusebi, 2010). The beginning of a new era in cancer research is marked by the development of methods to measure the expression of thousands of genes in tumour and in normal tissues. The regulation of gene expression occurs through different molecular mechanisms such as the transcriptional, transduction and post-transcriptional control. The first mechanism operates at the frequency of transcription of messenger RNA from its own template DNA. The transduction control regulates the speed with which a molecule of mRNA is translated into a protein. The post-transcriptional control occurs in the moment when modulation of protein synthesis is required, adjusting the speed or degrading a transcript that is already synthesized. The functionality of these systems enables the processing of complex gene expression profiles (Watson, 2008). Variation in transcriptional programs accounts for much of the biological diversity of human cells and tumours. In each cell, signal transduction and regulatory systems transduce information from the cell identity to its environmental status, thereby controlling the level of expression of every gene in the genome (Perou *et al*, 2000; Sorlie *et al*. 2001). The analysis of gene expression is the realization of the reform of tumour taxonomy, in predicting the metastatic potential, prognosis and response to therapy. It is also used to reveal patterns of gene expression that are dependent on the mutation of a single oncogene, and finally, in analyzing the effects of hormones and environmental influences on carcinogenesis (Robbins and Cotran, 2010).

Sorlie et al. (2001) laid the foundations for the new taxonomy demonstrating that changes in patterns of gene expression, analyzed by cDNA microarray techniques and hierarchical clustering, allowed a 'molecular portrait' to be defined for each tumour.

Recently, molecular characterization in human breast cancer has also been applied to the metastasing lymph nodes and to the systemic metastases (Wu *et al*., 2008).

As in Human Medicine, mammary gland tumours commonly occur also in female dogs (Gama *et al*., 2008) and cats (Morris *et al*., 2008), representing a remarkably heterogeneous group in terms of morphology and biological behaviour (Nielsen *et al*., 2004). In the last decades, veterinary attention has focused on the identification of reliable prognostic factors, such as tumour size, histologic type, histologic grade, lymph node status (Misdorp *et al*., 1999; Gama *et al.*2008; Sassi *et al.* 2010) and protein expression profile (Gama *et al*., 2008; Sassi *et al*., 2010), essential in order to estimate the individual risk of unfavourable clinical outcome (Misdorp, 2002; Zaidan, 2008). Gama *et al*. in 2008 and Sassi *et al*. in 2010 were the first who applied, from human medicine, an immunohistochemical algorithm and identified molecular phenotypes in canine mammary tumours.

Therefore, aims, developed as projects, of the past three years have been (1) to define the molecular phenotype of feline mammary carcinomas and their lymph node metastases according to a previous modified algorithm and to demonstrate the different relation between the primary tumour and lymph node metastasis, (2) to analyze, in female dogs, the relationship between the primary mammary tumor and its lymph node metastasis based on immunohistochemical molecular characterization in order to develop the most specific prognostic-predictive models and targeted therapeutic options, and (3) to evaluate the molecular trend of cancer from its primary location to systemic metastases cats and dogs with mammary tumors. Before the following research, no literature reports have addressed the immunophenotyping of feline mammary carcinomas.

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In order to make this study more complete, a parallel project has been carried out on the myoepithelial cells of canine mammary tumours. In canine normal mamamary gland, the lumina are delimitated by an inner layer of polarized epithelial cells resting on two outer or basal layers of epithelial and myoepithelial cells (Ramalho *et al*., 2006). Both basal and myoepithelial cells synthesize the basement membrane of ducts and alveoli and form a structural barrier between the luminal epithelial cells and the surrounding stroma (Polyak and Hu, 2006). The studies on mammary tumours, particularly in dogs, have drawn gradually increasing attention not exclusively to the epithelial component, but also to the myoepithelial cells. Myoepithelial cell proliferation is a frequent finding in the so-called complex and mixed patterns (Misdorp *et al*., 1999) but it is an uncommon feature of breast cancer in women (Sassi *et al*., 2010) and in cats. The lack of complete information on a valid panel of markers for the identification of these cells in the normal and neoplastic mammary gland and the lack of investigation of immunohistochemical changes from an epithelial to a mesenchymal phenotype were the aims of a parallel research started in 2012, and which has been included in the present report.

In addition, another study has been developed concerning CD117, a membrane-associated tyrosine kinase growth factor receptor encoded by *C-Kit* gene (Yarden *et al*., 1987). CD117 is expressed in various cell types during embryonic development, it promotes different functions of cells and has been shown to be expressed by neoplastic cells as well (Ronnstrand, 2004). Investigating mammary tumours, we noticed that only few studies had focused on the expression of CD117, on the difference in expression between normal tissue and neoplastic benign or malignant change in canine mammary tissue (Sailasuta *et al.,* 2008). Since *c-Kit* is a proto-oncogene that encodes a transmembrane tyrosine kinase growth factor receptor and stimulates cell proliferation, it plays a crucial role in determining the existence of a correlation between *c-Kit* expression and Ki67 index (Thompson *et al.,* 2011). Therefore, it was decided

to further deepen the knowledge in order to to characterize the immunohistochemical staining of CD117 in normal and neoplastic mammary tissue of the dog, and to correlate CD117 immunohistochemical results with mammary histotype, histological stage (invasiveness), Ki67 index and patient survival time.

The reader of this paper will find the findings acquired in the three years 2011-2013, which have been published or presented to national and international meetings.

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GENERAL SECTION

2. SOME INFORMATION ABOUT BREAST CANCER

Molecular taxonomy

The changes in gene expression patterns, using complementary DNA microarrays, provided a distinctive molecular portrait of each tumour (Perou *et al*, 2000). Sets of co-expressed genes were identified for which variation in messenger RNA levels could be related to specific features of physiological variation. The tumours were classified into subtypes distinguished by pervasive differences in their gene expression patterns of mammary epithelium (Perou et al, 2000). Based on those differences four subtypes were identified:

- Luminal like/ER+ (Estrogen Receprtor): so called because of the expression of many genes is expressed by the luminal cells.

- C-erb-B2: c-erbB-2 overexpression gene.

- Basal like: expression of a clusters of gene proper of the basal epithelium.

- Normal breast: overexpression of basal epithelium gene and low expression of genes of the luminal epithelium.

Subsequently, Sorlie *et al*. (2001) modified the molecular classification, in search for correlations between gene expression patterns and parameters of clinical relevance, such as the survival rate and the likelihood of recidivism, thus demonstrating the prognostic value of this subtyping. Two major branches of subtypes were obtained: ER- and ER+ (luminal). The ER- cluster included the basal-like subtype (characterized by high expression of cytokeratin 5 and laminin 17 binding fatty acids), the c-erbB-2 subtype (characterized by a wide expression of genes in the segment c-erbB-2), and the normal group breast-like (which showed the expression of genes typical of adipose tissue and other non-epithelial cell types) (Sorlie *et al*., 2001). These three clusters exhibited a remarkable expression of genes of the basal epithelium and a reduced or no expression of genes of the luminal epithelium. The second main group,

ER+, obtained from the analysis included three hierarchical sub- expressing genes typical of luminal epithelium, such as luminal A (high gene expression of ERα), luminal B and C (moderate expression of specific genes of luminal epithelium). In particular, the luminal C subtype was distinct from the other two luminal types for the expression of a set of genes whose function was unknown. The gene expression pattern represent the tumour biology, reflecting the biological diversity and correlate the different tumours with the clinical genomics was thought to be the key to understand this diversity (Sorlie *et al*., 2001).

Further refinement for molecular phenotyping was given by Sorlie *et al*. (2003) in a subsequent study, thanks to the increasing in number of tumour cases, from 115 to 534, and to the use of intrinsic genes. The modified molecular phenotypes classification came to the number of subtypes known at present: basal-like, c-erbB-2 overexpressing, two luminal-like (A and B) and normal-like (Sorlie *et al.*, 2003). The existence of different subtypes of breast cancer was confirmed by protein expression patterns assessed by immunohistochemistry (IHC) on tissue microarray (TMA), an efficient and reliable platform for subclassifying breast cancers into relevant subtypes, using a limited number of markers (Matos *et al*., 2005). In particular, the panels encompass at least anti- estrogen receptor (ER), anti-progesterone receptor (PR), anti- c-erbB2 and antibasal cytokeratin antibodies (CK 5/6 and 14) (Cheang et al, 2008; Kim *et al*., 2006). The obtained new classification was therefore, constituited by two hormone (estrogen (ER) and/or progesterone (PR) receptor positive types [luminal A (ER and/or PR+, c-erbB-2−), luminal B (ER and/or PR+, c-erbB-2+)], and three hormone receptor negative [c-erbB-2 overexpressing (ER/PR−, c-erbB-2+), basal-like (ER/PR−, c-erbB-2−, CK5/6 and/or CK14 and/or p63+), and normal-like (ER/PR−, c-erbB-2−, CK5/6 and/or CK14 and/or p63−)]. Even if much has been learned in the last few years about the molecular taxonomy, it is still in evolution and likely to change over the coming years (Cummings *et al*., 2011).

Prognostic value

Breast cancer outcome in women varies widely and prognosis is determined by the pathologic examination of the primary carcinoma and the axillary lymph nodes. In 2002 the American Joint Commitee on Cancer (AJCC) identified six major prognostic factors that were recognised as strongest predictors of death. The major prognostic factors were:

- Invasive carcinoma versus in situ disease. By definition, in situ carcinoma is confined to the ductal system and cannot metastatize while invasive carcinoma usually locally or distantly metastatize.

- Distant metastases. Cure is unlike, especially in hormonally responsive tumours and the type of tumour influences the timing and location of metastases.

- Lymph node metastases. In absence of distant metastases, it is the most important prognostic factor for invasive carcinoma. Approximally 10% to 20% of women without axillary lymph node metastases have a recurrence outside the breast and about the same number die from breast cancer. In these patients, metastasis may occur via the internal mammary lymph nodes or hematogenously.

- Tumour size. The size of a an invasive carcinoma is the second most important prognostic factor. The risk of axillary lymph nodes metastases increases with the size of the primary tumour, but both are indipendent of prognostic factors.

- Locally advanced disease. Carcinomas invading into skin or skeletal muscle are usually large and may be difficult to treat surgically.

- Infllammatory carcinoma. Particularly poor prognosis is related to breast cancer presenting with breast swelling and skin thickening due to dermal lymphatic involvement.

In addition to the six major factors used by the AJCC, a number of other factors have become increasingly important as predictive of outcome.

- Histologic Subtype. The survival rate of women with special type of invasive carcinomas (tubular, mucinous, medullary, lobular, and papillary) is greater than 60%, compared with less than 20% for women with non special type.

- Histologic grade. The commonly used grading system, the Nottingham Histologic Score, combines nuclear grade, tubule formation and mitotic rate to classify invasive carcinomas into three groups that are highly correlated with survival. A higher survival for patients with well-differentiated grade 1 carcinoma, and, in contrast, most deaths for poorly differentiated grade 3 carcinomas.

- Estrogen and progesteron receptors. Immunohistochmistry assays are currently used to detect nuclear hormone receptors. This latter finding is correlated with a better outcome and is an important predictor of response to hormonal therapy.

- C-erbB-2. Its overexpression is associated with poor survival, but its main importance is as a predictor of response agents that target this transmembrane protein (trastuzumab or lapatinib).

- Lymphovascular invasion. The presence of tumour cells within vascular spaces is strongly associated with the presence of lymph node metastases. It is a poor prognostic factor for overall survival in women without lymph node metastases and a risk factor for local recurrence.

- Proliferative rate. Carcinomas with high proliferation rates have a poorer prognosis but may respond better to chemotherapy. The rate can be measured by mitotic counts or by immunohistochemical detection of cellular protein produced during the in cell cycle (ki-67).

- DNA content. Tumours with abnormal DNA indices have a slightly worse prognosis.

- Response to nonadjuvant therapy. Pathologic response to chemotherapy can be used as a short-term end point for clinical trials

- Gene expression profiling. Expression profiling has been shown to predict survival and recurrence-free interval, and also identifies patients who are most likely to benefit from particular types of chemotherapy (Robbins and Cotran, 2010)

Standard clinical prognostic factors, such as patient age, tumour size, lymph node status, tumour grade, hormone receptor status or c-erbB-2 provide valuable information on the risk of recurrence; however, these estimates of clinical risk are coarse. On the contrary, multigene classifiers have the potential to complement traditional methods through provision of additional biological prognostic and predictive information in presently indeterminate risk groups (Rakha and Ellis, 2011). Indeed, the different molecular subtypes have been associated with different prognoses (Sorlie *et al*., 2003), treatment planning (Carey, 2011), metastatic sites (Fountzilas *et al*. 2012; Gabos *et al*. 2006) and survival rates (Blows *et al*., 2010). Sorlie *et al.* (2001), based on a study of 49 patients with locally advanced disease status but without systemic metastases, conducted a survival analysis correlating ther subtypes with the overall survival rate and recurrence-free interval . The recurrence-free interval was defined as the interval between the date of breast surgery and the date of subsequent diagnosis of breast cancer. The overall survival rate is defined as the interval between the date of breast surgery and the date of death, related to breast cancer (Sotiriou *et al*., 2003). The added value identified by Sorlie *et al*. (2001) gave a clinical significance to these tumour subtypes, demonstrating a low survival rate and a high relapse rate for the Basal-like and c-erB-2+ subtypes, accompanied by a poor prognosis, with a high frequency of mutation in the TP53 gene and amplification of the c-erbB-2 gene, respectively. The luminal presented a more

favorable prognosis than the previous   but with different outcomes between luminal A and B. The latter showed a worse outcome than the first (Sorlie *et al.,* 2001).

The molecular processes at the basis of tumour progression have not been fully characterized as well as the factors that determine the metastatic potential of a tumour, both to the respective regional lymph node or to distant sites. A common hypothesis argues that the metastatic process is affected by the molecular changes that evolve within few clones of the primary tumour that metastasize. If this were true, the metastasizing ability of a small proportion of the primary tumour clones could not be predicted by the analysis of the primary tumour. An alternative hypothesis argues that the metastatic capacity is largely determined by the sum of the molecular changes that characterize the majority of cells in the primary tumour. In this case, the metastases could be predictable. The status of the regional lymph node, determined at the time of diagnosis, may represent a functional, even if imperfect surrogate of the process of metastasis. Lymph node metastases reflect the biological properties of the tumour (eg, motility, invasiveness) (Lu *et al.*, 2008).

The concept that the molecular information of the primary tumour may help   define the metastatic potential was investigated by Lu *et al.* (2008). Their formulated models, constructed on the basis of gene expression data, demonstrated predictive accuracy to biological characteristics, such as the status of ER, c-erbB-2, and tumour grade. Instead, the model failed to demonstrate the predictive accuracy to anatomical features such as lymph node status, tumour size and lymph-vascular invasion. The authors concluded by saying that the regional metastases develop in relation to time and to a "propensity" that could be due to inherent biological differences, best reflected by molecular subtypes, and that their prediction is of limited value (Lu *et al.,* 2008).

A further merit that was given to the molecular analysis methods was the possibility to establish the preferential sites of distant metastases in association with different molecular

subtypes. It was found that bone metastases were more consistently associated with the luminal subtypes, less frequently with the basal phenotypes. The opposite was true for metastases to the lung and brain, with no specific correlation between lung metastases and luminal A type. The pleural metastases were found almost exclusively in the luminal subtypes (Smid *et al.*, 2008). The ability of the tumour to head, survive and proliferate in certain distant organs requires a different set of genes, as well as the ability of the tumour to metastasize. A lot of genes differentially expressed were identified, many of which were common among the subtype and the site in which the subtype had preferentially metastasized; wingless-type MMTV integration site family (WNT) signalling was up-regulated in the basal subtype as well as in its specific brain metastasis. The five molecular subtypes are therefore clearly different with regard to their ability to metastasize to different body sites (Smid *et al.*, 2008).

The profile of gene expression is thus a most powerful predictor of the outcome of the disease, than traditional systems based on clinical and histological criteria (van de Vijver *et al.*, 2002).

Target therapeutic and predictive approach

While prognostic factors intend to objectively predict the clinical outcome independently from treatment, the predictive approaches are intended to predict the response of patiens to specific therapeutic therapies and are also associated with tumour sensitivity or resistance to therapy (Weigel *et al.*, 2010).

The discovery of molecular subtypes of breast cancer gave a further proof of the fact that biological diversity consequently denies a unique therapeutic approach (Peppercorn *et al.*,

2008). The therapeutic approach to breast cancer varies according to the different molecular phenotypes.

The luminal subtypes are hormone receptor-positive, therefore appropriate for endocrine therapy with tamoxifen combined with chemotherapy (Peppercorn *et al*., 2008). This is especially true for the luminal A, in which conversely only a small amount of those tumours respond to classical chemotherapy. The luminal B, also referred to as triple positive for the expression of c-erbB-2 as well as hormone receptor, is often responsive to chemotherapy (Robbins and Cotran, 2010).

The expression of ERα is lately undoubtedly considered the most important biomarkers in breast cancer, because it provides an index of sensitivity to endocrine treatment (Weigel and Dowsett, 2010).

The therapeutic approach to c-erbB-2-overexpressing phenotypes is by treating patients with chemotherapy, and c-erbB-2 targeted therapy such as trastuzumab (Carey, 2011).

The c–erbB-2 overexpressing phenotypes, besides being a predictor factor of resistance to endocrine therapy or selective resistance to tamoxifen predicts resistance to some chemotherapies (Harris *et al*., 2007).

The therapeutic approach to basal-like and normal-like phenotypes represent today a huge challange since they are not responsive to hormone therapy, but they are treated with chemotherapy alone (Carey, 2011; Matos *et al*., 2005). Treatment of these phenotypes only with chemiotherapy implies an estimate of the residual risk of 30-40% (Fig. 1) (Carey, 2011).

Fig. 1: Estimate residual risk
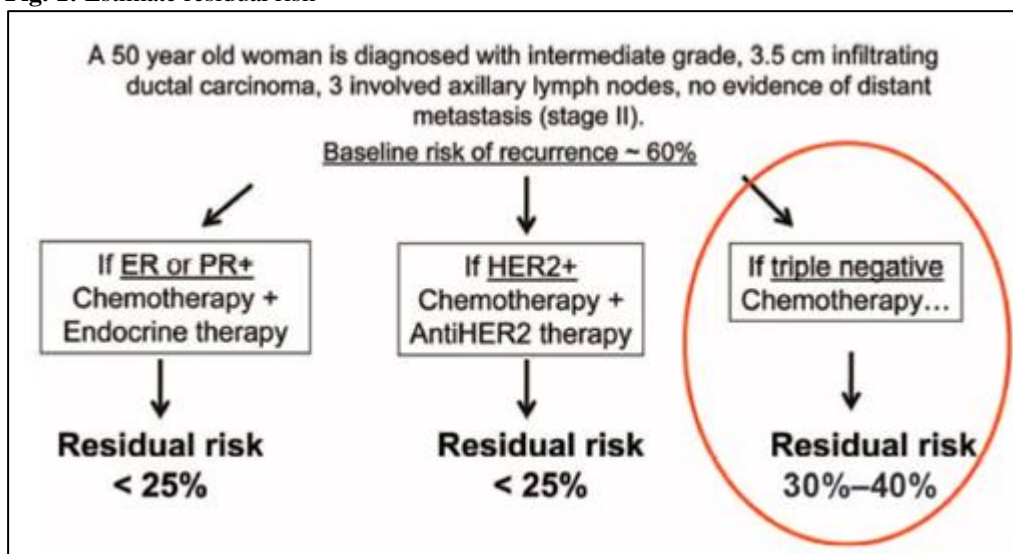
Adjuvant therapy for breast cancer in the initial state, with the initial risk of recurrence of approximately 60%. If the patient present a tumour hormone receptor positive phenotype, she will be treated with endocrine therapy and chemotherapy, with a subsequent residual risk of recurrence <25%. In case of c-erbB-2 overexpressing phenotype (c-erbB-2+), the patient will be treated with anti- c-erbB-2 therapy in addition to chemotherapy, with estimates residual risk of <25%. If the cancer present a triple negative phenotype the treatment is chemotherapy only, with residual risk> 30-40% (Modified from Carey, 2011).

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3. PHENOTYPIC CONCORDANCE AND DISCORDANCE BETWEEN PRIMARY MAMMARY CARCINOMA AND ITS RELATED METASTASES

The study by Weigelt *et al.* (2003) supported the concept that the ability to metastasize to distant sites is a genetic properties of breast carcinomas. Shown that the gene expression profile of the primary tumour is closely similar in distant metastases of the same patient, indicative of the hypothesis that the ability of the metastatic breast cancer is an innate characteristic and it is not based on a clonal selection (Weigelt *et al.*, 2003).

Previously, Fidler *et al.* (1977) found that clones, obtained in vitro from a parental malignant melanoma murine cell culture, varied greatly in their ability to produce metastatic colonies. This involved the striking conclusion that the sub-cell population having highly metastatic power pre-existed in parental population (Fidler *et al.*, 1977). This model implies that metastases arise from a sub-clone, which could be molecularly distinct from their primary tumour. The Fidler clonal concept is widely accepted, although the metastatic process has also been described as a stochastic event, having the primary tumour cells with equal metastatic potential (Weigelt *et al.*, 2003; Milas *et al.*, 1983).

Many human tumours develop through a succession of genetic and epigenetic changes that confer to the cells neoplastic characteristic. This process can be likened to a Darwinian evolution within the microcosm of living tissues, in which individual cells represent the selection unit. Single cells that possesses advantageous characteristics (such as survival and proliferation) are selected to become the progenitor of a successor cell population that eventually will dominate the tumour mass. The initiator of the next round of successors clones represent a rare variant that arises between the many successor cells. About 6-10 cycles of clonal sequences are needed to generate highly malignant tumour cells. A different model of

metastatic mechanism relies on the fact that the tendency to metastasize is largely determined by the identity of the mutant alleles, which are acquired relative at the beginning of the various carcinogenesis stages (Bernards *et al*., 2002).

In order to investigate the molecular differences between the primary tumour and metastases Ramaswamy *et al*. (2003) compared the gene expression profile of metastatic adenocarcinoma form multiple tumour phenotypes with not corresponding primary adenocarcinomas. They proved that a part of primary tumours resembled the metastatic ones regarding the gene expression signature (Ramaswamy *et al.*, 2003).

Weigelt *et al*. (2005), studying the gene expression profile of a set of 70, already proved to be efficacy in predicting the metastatic potential, found that distant metastases reflect the molecular subtypes and prognostic signature (70 genes) of their primary tumour. These results support the hypothesis that the molecular subtypes originate from different cell types within the mammary gland and thus reflect the different biological entities, which are kept in the metastatic process (Weigelt *et al*. 2005).

Divergent results were deduced from a study of Aitken *et al*. (2010), which carried out changes in expression through quantitative analysis of ER, PR, c-erbB-2 in primary mammary site tumour and its related lymph node metastasis. Given that nodes are excised in standard surgical practice, as sentinel node biopsy, axillary node sample, or node clearance, and assessed by a pathologist for routine staging, the authors showed that there may be added benefit to molecular testing on nodal metastases as well as the primary tumour in order to guide adjuvant therapy. While this might incur additional financial costs for specimen processing and molecular analysis, savings could be made by avoiding overtreatment. The authors demonstrated that a significant number of patients showed a quantitative difference in expression of the molecular markers between primary tumour and lymph node metastasis and this data may confer increase therapeutic sensitivity or resistance to targeted therapy.

Therefore, the difference in receptor expression levels may be one of the causes of treatment failures, found in some clinical cases and the phenotyping of both primary and lymph node would reduce morbidity for patients and ultimately has the potential to produce more favourable clinical outcomes (Aitken *et al*., 2010).

In conclusion the different studies regard the comparison between the phenotypes of primary site and lymph node metastasis have highlighted the existence of a concordance in which the primary tumour and the lymph node metastasis have the same phenotype, and a discordance with differences in phenotype between primary and metastases. On the basis of this knowledge, the future treatment decisions should be based on the gene expression profile of both the primary tumour and its related lymph node metastasis (Aitken *et al*., 2010).

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4. MOLECULAR PHENOTYPES IN CANINE MAMMARY TUMOURS

Mammary tumours are among the most common tumour in dogs, in fact, they represent 25–50% of all neoplasms in this species, with an average age of occurrence between 6 and 10 years (Millanta *et al*., 2005a).

The high homology between the canine and the human genomic sequence, as the many similarities regarding the morphology, biological behavior, and clinical course of mammary tumours, is the starting point for the study of comparative pathology that is proposing the dog as a valuable comparative and predictive model for human breast cancer. Recent studies have shown that the general basic biology of cancer in dogs such as tumour establishment and metastatic progression is similar to what happens in human cancer (Pinho *et al*., 2012). Most of the cancer-associated genetic alterations (Fig. 2) that are known to play a role in mammary tumour development and progression are similar in both species (Rivera *et al.,* 2011).

A recent study demonstrated that many genes deregulated in human breast cancer were also found deregulated in canine mammary tumours compared with normal mammary tissue. This first genome-wide comparative analysis demonstrated that the pathways showing upregulation in tumour from both species are those related to increased proliferation activity, whereas the pathways related to cell development, cell matrix adhesion, and cell communication are downregulated. Furthermore, it has also been shown a great degree of homology between human and canine mammary tumours in the perturbation of many cancer related pathways (Uva *et al*., 2009). The genome similarities of dogs and humans support strongly the genetic homology between both species (Pinho *et al*., 2012).

Fig. 2: Comparative analysis of the role of the critical genes and signaling pathways involved in the carcinogenesis of human breast cancer and canine mammary tumour

	Human Breast Cancer	Canine Mammary Tumor
Gene sets/signaling pathways		
PI3K/AKT	Upregulation[21-25]	Upregulation[21-23]
KRAS	Upregulation[21,26]	Upregulation[21,26]
PTEN	Downregulation[21,22]	Downregulation[21,27]
Wnt-β catenin	Upregulation[21,30]	Upregulation[21,31]
MAPK cascade	Upregulation[21,32]	Upregulation[21]
BRCA1	Downregulation[21,33-36]	Downregulation[21]
BRCA2	Upregulation[21,33-36]	Upregulation[21,37,38]
p53	Downregulation[39]	Downregulation[40-42]
Steroid hormone receptors		
ERA	Upregulation[43-45]	Upregulation (controversial)[46-48]
PR	Downregulation (controversial)[43-45]	Downregulation (controversial)[46,47]
Other cancer-related molecules		
E-cadherin	Downregulation[49,50]	Downregulation[49,51-55]
Sialyl Lewis x	Upregulation[49]	Upregulation[49,53]
P-cadherin	Upregulation[56,57]	Upregulation[58]
EGRF	Dysregulation[59,60]	Controversial[61,62]

The first study regarding the gene expression of mammary tumours was accomplished by Rao *et al.* (2008) who characterized, with the use of the cDNA microarray, the expression profile of three different cell lines of canine mammary carcinoma originating from histologically distinct primary tumours:

- CMT (Canine Mammary Tumour) - U335 (histological type osteosarcoma)

- CMT- U229 (atypical benign in mixed tumour histological type)

- P114 (anaplastic histological type carcinoma)

The evaluation of the gene expression profile of the cell lines showed correspondence with the tumour of origin and the differential regulation of several pathways such as the WNT, integrins, cell cycle, alternative complement cascade, cytokine/Rho-GTPase. These pathways showed overlaps with those found in humans, therefore the expression profile of spontaneous canine mammary carcinomas was supposed to act as a biological sieve for the identification of pathways or of gene expression profiles that are involved in carcinogenesis (Rao *et al.*, 2008). The highlighting of molecular subtypes of canine mammary tumours by using gene expression patterns of a intrinsic gene set, similarly to what was done in humans, has not been

performed in veterinary medicine because of a reduced representation of similar genes on the canine microarray (*Rao et al.*, 2008).

The new classification of distinct molecular phenotypes was developed by Sarli *et al.* (2007) using an immunohistochemical panel with anti-cytokeratin 19 (CK19), -CK14, -CK 5/6, -ER, -PR, -vimentin, -c-erbB-2, in order to identify the following phenotypes:

• luminal -like (CK19+, ER+/-, PR+/-, CK14-, CK5/6-) type A (c-erbB-2-) and type B (c-erbB -2+);

• basal- like (CK19-, ER-, PR-, CK14+, CK5/6+, c-erbB-2-);

• c-erbB-2+ (CK19-, ER-, PR-, CK14-, CK5/6-, c-erbB-2+).

The subtypes luminal A and luminal B showed the same phenotype but differed in the expression of c-erbB-2, which is considered an important prognostic index of tumour progression in canine mammary tumours as well as in woman breast cancer. The myoepithelial proliferation, in complex and in mixed tumour, resulted negative for myoepithelial cells markers (CK14 and CK5/6), which characterize the basal- like phenotype, but were positive for vimentin. The positivity for cytokeratins was expressed only when the cells were surrounded by the myoepithelial luminal counterpart. Sarli *et al.* (2007) suggested that the above mentioned data could be indicative of lack of specific phenotypes in canine pathology or of the need to investigate further for myoepithelial markers.

On the basis of the immunohistochemical panel proposed by Matos *et al.* (2005), Gama *et al.* (2008) characterized 102 canine mammary carcinomas based on the immunohistochemical panel which involved the evaluation of five molecular markers (ER, c-erbB-2, CK5, p63 and P-cadherin). ER and p63 positive cases showed the characteristic nuclear staining, whereas those positive for CK5 showed a cytoplasmic pattern. The c-erbB-2 positive tumours to have presented a membranous staining, P-cadherin positive tumours showed both a cytoplasmic or a membranous staining. The tumour classification was effected on the basis of ER and c-

erbB-2 status, in accordance with the algorithm formulated by Nielsen *et al.* (2004); positive tumours to ER were classified as luminal, distinguishing them further into A and B on the basis of negativity or positivity to c-erbB-2, respectively. Tumour that did not express ER, but were positive for c-erbB-2 were defined as c-erbB-2 overexpressing phenotype, or those cases that continued to be negative but positive for basal markers CK5, p63, and P-cadherin were classified as basal. Tumours that did not express any markers were identified as "none phenotype". CK5, p63 and P-cadherin are proteins that are expressed early in epithelial differentiation and may contribute to a committed stem cell and/or progenitor phenotype (Boecker and Buerger, 2003; Boecker *et al.,* 2002). In the study by Gama *et al.* (2008), the authors demonstrated that these markers were upregulated in the basal subtype, similarly to the previous results by Matos *et al.* (2005). In fact, the basal subtype rarely expressed just one basal marker but frequently expressed them simultaneously, which suggested a more undifferentiated profile. C-erbB-2 overexpressing subtype was also characterised by an up-regulation of basal markers, confirming some human breast studies, which suggested that c-erbB-2-overexpressing tumours should be included in a bona fide basal-like subclass (Gama *et al.*, 2008; Matos *et al.* 2005). In contrast, the majority of luminal tumours in Gama *et al.* (2008) series were simultaneously negative for basal cell markers, with some cases showing basal marker expression, which was also described by some authors who reported co-expressing basal CK and hormone receptors or c-erbB-2.

The correlation between subtypes obtained and histology showed that ER positive luminal A tumours more frequently associated with complex tumour type, low histological grade, less invasive and low proliferative tumours (Gama *et al.*, 2008; Sorlie *et al.*, 2003), whereas basal-like and c-erbB-2 overexpressing subtypes were associated with simple and carcinosarcoma tumour types, high histological grade, lymphovascular invasion and high proliferation,

features that are in accordance to the ones described in human literature for basal-like cancers (Gama *et al*., 2008; Kim *et al*., 2006; Matos *et al*., 2005; Sorlie *et al*., 2001).

In terms of prognosis, it is already well known that molecular phenotypes are associated with different clinical outcomes in canine mammary tumours as well as in human tumours (Gama *et al*., 2008; Sorlie *et al*., 2003). Basal subtype is associated with lower survival rates and appearence of recurence, similarly to human breast cancer studies. In contrast to basal subgroup, luminal and c-erbB-2 overexpressing subtypes show increased survival rates.

The fact that luminal tumours were associated with a better prognosis is not surprising since ER positive human breast carcinomas are usually associated with a more favourable clinical outcome. In veterinary pathology, however, the prognostic value of ER in canine mammary cancer is still a matter of debate. C-erbB-2 overexpressing tumours were found usually associated with established indicators of poor prognosis such as large tumour size, high histologic grade, invasion, simple histologic type and high proliferative indices (Gama *et al*., 2008; Rakha *et al*., 2006; Sorlie *et al*., 2008). However, Kaplan–Meier analysis in the study by Gama *et al*. (2008) revealed that C-erbB-2 subtype was related with a more favourable clinical outcome, findings that were in contrast with human studies, which describe similar survival rates for c-erbB-2 overexpressing and basal-like subtypes (Gama *et al*., 2008; Rakha *et al*., 2006: Sorlie *et al*., 2008).

In order to evaluate the prognostic potential of the hormone receptors expression in canine malignant mammary tumours, Chang *et al*. (2009) conducted a study on benign and malignant tumours. The expression of ER and PR was significantly more frequent in benign tumours compared to the malignant counterpart. Moreover, among the malignant samples only those expressing ER and PR had a high survival rate, compared with malignant tumours expressing ER but not PR suggesting, therefore, that the PR could be considered as an important prognostic factor (Chang *et al*., 2009).

It is known that malignant tumours negative to PR receptors have a higher proliferation index compared to those PR+, suggestive of the fact that the progression to malignancy in spontaneous mammary tumours was accompanied by a decrease in the dependence hormonal steroid (Geraldes *et al*., 2000).

In the study by Millanta *et al*. (2005), the expression pattern of canine steroid receptors showed that ER expression is significantly high in the normal tissue, in hyperplastic and dysplastic lesions, and in benign tumours, but significantly lower in carcinomas. There were not apparent significant changes in the reactivity of PR in normal tissue, dysplastic and benign neoplastic lesions except for a significant reduction of expression in carcinomas. The absence of ER and PR only in the female dog is associated with a high mortality rate and these data confirm previous observations that demonstrated that canine mammary tumours have features in common with hormone-dependent human breast cancer (Millanta *et al*., 2005a)

The immunohistochemical investigation of ER in canine mammary tumours was found to be a simple application technique with prognostic value that could be useful for appropriate hormonal therapies selection (Nieto *et al*., 2000).

With regard to the prognostic value of c-erbB-2, studies present in literature reported controversial data, in spite of what is known in the woman. According to Hsu *et al*. (2009), the relationship between the clinical course and the protein expression of c-erbB-2 in dogs with malignant mammary tumour indicated a greater overall survival rate in c-erbB-2 overexpressing tumours compared to those with normal levels of the antigen. The reason for such a difference between c-erbB-2 overexpressing phenotype in canine and in women breast cancer remained unclear for the authors. Certainly c-erbB-2 seemed to play an important role in tumour formation, but did not seem to be directly correlated with the progression to malignancy (Hsu *et al*., 2009). Given the high expectation of suraival rate in canine mammary

c-erbB-2+ tumours, the role of this protein in oncogenesis may be different from that played in breast cancer (Hsu *et al*., 2009).

Opposite results results exerted that c-erbB-2+ tumours were correlated with indicators of poor prognosis, such as tumour size >3cm, histological grade III [presence of neoplastic emboli (Gilbertson *et al*., 1983)], type of invasive growth, absence of hormone receptors and a period of <6 months without relapse after surgery. Those results were in agreement with what has been observed in the human, having characteristics indicative of a worse prognosis (Martin de las Mulas *et al*., 2003; Sorlie *et al.*, 2003).

Mammary tumours in the dog are characterized by significant high molecular heterogeneity, thereby not surprising that individual markers could not accurately estimate the heterogeneity of breast cancer (Rakha *et al*., 2009) and that, on the contrary, could benefit from a classification based on their molecular differences.

Sassi *et al*. (2010) applied an immunohistochemical panel (anti –ER, -PR, c-erbB-2, -CK5/6 and -CK14) to a series of canine mammary carcinomas in order to: identify molecular phenotypes based on a modified molecular classification, find possible correlation between the phenotypes and stage and histological grade, and use the phenotypes as a prognostic aid in veterinary practice. The molecular phenotypes have been identified in accordance with the flowchart classification proposed by Conforti *et al.* (2007) (Fig.3).

Fig. 3: Flowchart classification based on Sassi *et al.* (2010)

Figure 1 Classification flowchart followed in the present study (from Conforti et al. [7] modified).
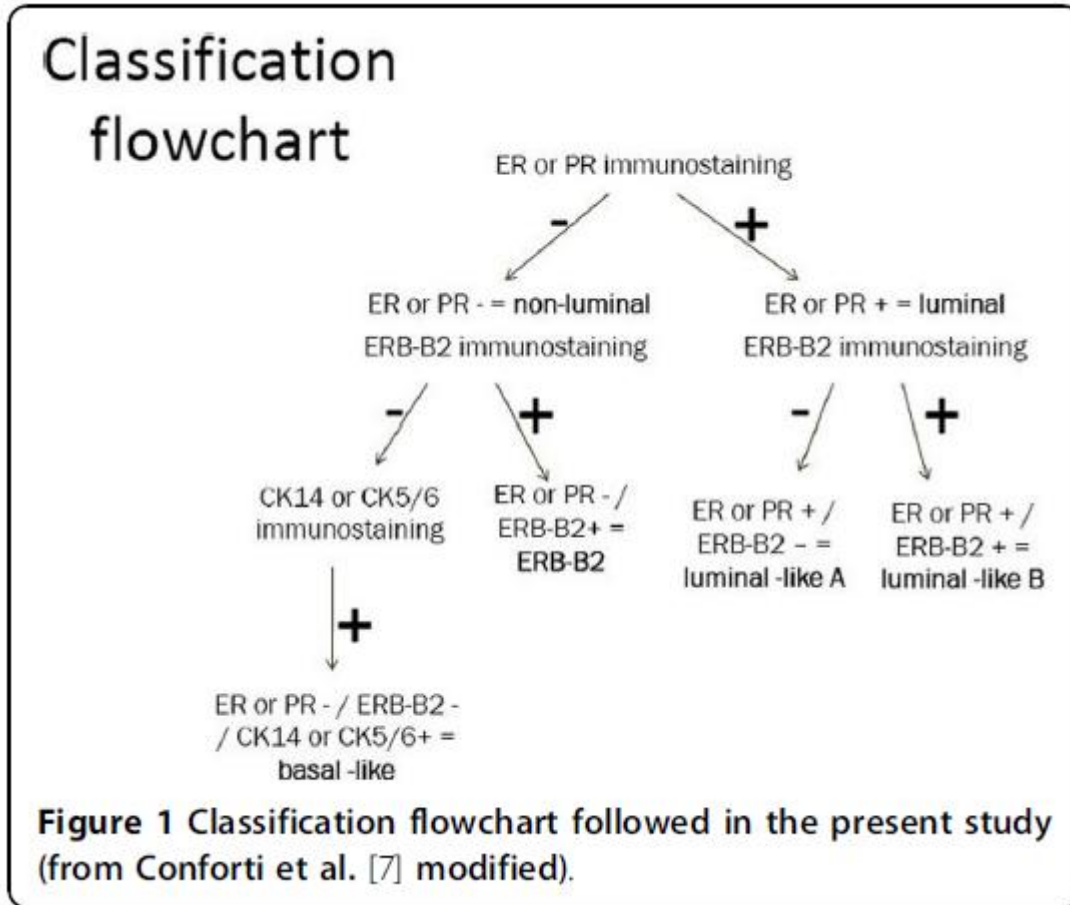
Out of 45 samples, the panel of antibodies has identified only three tumour groups (luminal A, luminal B and Basal-like) out of the five groups known in Human Medicine (Sassi *et al.*, 2010) and still different from the four identified (luminal A and B, Basal-like, c-erbB-2 overexpressing) found by Gama *et al.* (2008). The uses of different panels and criteria to define markers positivity was probably the cause of the differences found between Gama *et al*. (2008) and Sassi *et al.* (2010) studies. Sassi *et al*. (2010) demonstrated a correlation between the molecular based classification and the histological grade, but they did not find a relation between the stage and the morphological classification of tumours. In addition, luminal A phenotype included a high percentage of grade I tumour, compared to luminal B in which prevailed grade II and III tumours. On the contrary, Gama *et al*. (2008) showed that only the basal-like phenotype was correlated with grade and vascular invasion. Sassi *et al*.

(2010) did not detect any similarity between the molecular classification system and survival rates, while Gama *et al*. (2008) found only for the basal phenotype the association with short survival.

In multivariate analysis, according to Sassi *et al*. (2010), staging and histological grade showed an independent association with survival rate, while the phenotypes and the histological types did not show any association. These data suggested that caution should be used when applying the new classification system for canine mammary tumours, in which the fundamental prognostic information derived from the staging and histological grade (Sassi *et al*., 2010).

Canine mammary tumours have been treated in different ways with surgery as the first choice of therapy, either alone or in combination with chemotherapy (Lana *et al*., 2007) even though no standard therapeutic protocols are available (Lavalle *et al*., 2012). Receptor evaluation has been introduced to use an anti-estrogen therapy, whose side-effects include endometritis in female dogs with ER negative tumours (Morris *et al*., 1993)

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5. MOLECULAR PHENOTYPES IN FELINE MAMMARY TUMOURS

Mammary tumours are common in female cats, especially in older animals, and the majority of them are malignant and very aggressive (MacEwen and Withrow 1996; Multon, 1990). Feline malignant tumours grow rapidly, and metastases are reported to occur in 50–90% of affected animals (Hayden and Nielsen, 1971). Metastasis to regional lymph nodes (83%), lungs (83%), pleura (22%) and liver (25%) are most common. However, various studies have also documented widespread metastases to the adrenal glands, diaphragm and kidneys (Giménez *et al*., 2010).

Nowadays, the most important prognostic factor in cats with mammary gland neoplasia is tumour size, which significantly affects both disease-free interval and survival time (Lana *et al*., 2001). Other important prognostic factors are:

- histopathologic grade: An association between survival time and histopathologic grade has been demonstrated. The rate of death 1 year after surgery was 0% in cats with well-differentiated carcinoma, and 100% in those with poorly differentiated carcinoma. However, there was not a good correlation between moderate differentiation and survival time (Jeglum *et al*., 1985);

- mitotic rate: The number of mitotic figures found in tumour tissue has been shown to be of prognostic value. Longer survival times were seen in animals with tumours exhibiting fewer than two mitotic figures per high power field (Weijer *et al.*, 1983);

- disease stage: Clinical stage at presentation has been shown to be significantly associated with survival time (Ito *et al*. 1996);

- surgical approach: In a study of cats bearing malignant mammary adenocarcinoma, which had undergone radical mastectomy, a significantly

reduced rate of local recurrence was compared with cats that had undergone conservative surgery (MacEwen *et al.*, 1984);

- molecular markers: The expression of some genes, receptors or proteins can be altered during the malignant process in feline mammary tumours. These so-called molecular markers can be detected by immunohistochemistry and yield useful prognostic information in some cases (Giménez *et al.*, 2010). Most studied markers are: hormone receptors ER and PR (Millanta *et al.*, 2006), c-erbB-2 (Millanta *et al.*, 2005b), cyclin A, protein p53, macrophage-stimulating protein receptor (RON), vascular endothelial growth factor (VEGF), cyclo-oxygenase (COX) and topoisomerase IIβ binding protein 1 (TopBP1) (Giménez *et al.*, 2010).

Regarding the hormone receptors state, Martín de las Mulas *et al.* (2002) demonstrated through the analysis of the immunohistochemical expression of PR receptors in normal, dysplastic and neoplastic mammary glands of cats that benign mammary lesions, like those in dogs, appeared to have higher PR receptor profiles than mammary carcinomas, with 66.7% of benign tumours and dysplasias and 37.5% of malignant mammary lesions being progesterone receptor positive. The elevate percentage of feline PR+ tumours carcinomas suggested the possible role of PR as an early promoter of tumour growth in the cat (Millanta *et al.*, 2006). In 2000, Martín de las Mulas *et al.* considered the expression of ER, detecting significant decrease in ER positivity between normal and dysplastic tissues and invasive carcinomas. It has been suggested that steroid hormones may play a role in promoting cellular proliferation in the early stages of development of canine mammary tumours (Rutteman, 1990). Cellular proliferation has been recognised as a prognostic indicator in ER−human breast carcinomas, whereas benign mammary lesions in both cats and dogs (Rutteman *et al.*, 1993) had higher ER profiles. Estrogens might act by binding ER normally present in epithelial and stromal cells of mammary gland inducing in these cells an increased expression of PR receptors. In

this hypothesis the un-reactivity of epithelial and stromal cells for ER could be explained with a previous presence of ER in these tissues in the initial stage of the diseases and a progressive loss during the evolution of the disease (Millanta *et al*, 2005a). The high negative rates of ER expression seem to be a characteristic feature of feline mammary carcinomas when compared with human and canine tumours and suggest a lack of estrogen dependency as previously proposed. Also, the reduced percentage of ER+ invasive carcinomas demonstrate the behavior and phenotype of typically aggressive mammary tumour in cats (Martín de las Mulas *et al*., 2000). The evaluation of ER receptors state in normal, dysplastic and neoplastic feline mammary tissue assumed a prognostic value as ER expression was significantly higher in normal and hyperplastic tissue more than in neoplastic tissue. In light of the correlation between the hormone receptors expression and the proliferation index obtained by immunolabeling with MIB-1 (Mouse Monoclonal IgG1), ER+ phenotypes had a proliferation index significantly lower than ER- phentypes, having therefore a poorer prognosis (Millanta *et al*., 2006). Considering both estrogen and progesterone receptors, feline mammary carcinomas showed features in common with hormone-independent human breast cancer (Millanta *et al*., 2005a)

The feline c-erbB-2 was found to be barely detectable in normal mammary gland, increased in mammary benign tumours, and elevated in a high percentage in carcinomas. The correlation between c-erbB-2 overexpression and the overall survival rate showed that cats affected by c-erbB-2 overexpressing phenotype tumours had shorter overall survival time. These findings suggested that c-erbB-2 status could provide valuable prognostic and predictive information (Giménez *et al*., 2010). The effect of c-erbB-2 overexpression in feline mammary tumours seemed to be more similar to human tumours than in those of the dog (Millanta *et al.*, 2005b).

Treatment options that have been studied for feline mammary neoplasia are surgical excision, chemotherapy, immunotherapy and radiation therapy. These differ in terms of clinical outcome (Giménez *et al*., 2010; Lana *et al*., 2007). Regarding the chemiotherapy, a recent retrospective study on the use of adjuvant doxorubicin therapy after surgical excision in 67 cats reported a Kaplan Meier median survival time of 448 days and called for randomized trials to prove the true efficacy of chemotherapy (Novosad *et al*., 2006).

As for canine mammary carcinomas, also for queens no standard therapeutic protocols are available (Lavalle *et al*., 2012)

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EXPERIMENTAL SECTION

1. MOLECULAR PORTRAIT-BASED CORRELATION BETWEEN PRIMARY CANINE MAMMARY TUMOUR AND ITS LYMPH NODE METASTASIS: POSSIBLE PROGNOSTIC-PREDICTIVE MODELS AND/OR STRONGHOLD FOR SPECIFIC TREATMENTS?

RESEARCH ARTICLE Open Access

Molecular portrait-based correlation between primary canine mammary tumor and its lymph node metastasis: possible prognostic-predictive models and/or stronghold for specific treatments?

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Introduction

Canine mammary tumour and human breast cancer are heterogeneous diseases commonly occurring in female dogs [1,2] and in women [3,4].

Traditionally, breast cancer has been classified by morphological criteria in both human [5] and veterinary [6,7] medicine. Recent veterinary attention, as widely explained in the general part regard canine mammary tumours, has focused on the protein expression profile and four main carcinoma subtypes have been identified [8,9]: luminal A, luminal B, c-erbB-2 overexpressing and basal-like. Recently, molecular characterization in human breast cancer has also been applied to the metastasing lymph nodes [15]. The metastatic process is in fact the most urgent, important and difficult issue to approach in human [16] and animal cancer medicine. The relationship between the primary tumour and the lymph node metastasis from

the same patient was studied by Wu *et al.* [15] to determine if satellite tumour are uniform or divergent in molecular properties and to provide new information of diagnostic and therapeutic significance [16].

Recent publications emphasized several similarities between human breast cancer and canine mammary tumour, such as the relative age at onset, incidence, risk factors, biological behaviour, metastatic pattern, histopathological and molecular features, metastases-associated expression profile [17], and response to therapy [18,19].

The aim of this study was to analyze the relationship between the primary mammary tumour and its lymph node metastasis based on immunohistochemical molecular characterization to develop the most specific prognostic-predictive models and targeted therapeutic options.

Methods

Samples

Specimens of mammary carcinomas from 20 female dogs were collected from the database of the Pathology Service of the Department of Veterinary Medical Science of Bologna University and from the Department of Animal Pathology of Pisa University.

The 20 dogs comprised 11 mixed breed, two German Shepherd, one Yorkshire, two English Setter, two Doberman, one Maremma Shepherd and one Poodle. Dog ages ranged from six to 14 years with a mean age of 10.3 and a median of 10.5. Two-year follow-up survival data were available for 11 out of the 20 animals with invasive carcinomas included in the study. Overall survival time was defined as the time from the day of diagnosis until the day of death or last follow-up. All the latest data are summarized in Table 1.

Cases were selected based on both the primary mammary neoplasia and histological grade II (grade II: invasive carcinoma and metastases to regional lymph nodes) according to

Gilbertson *et al*. [25]. No cases displayed systemic metastases. Samples were available as sections stained with hematoxylin and eosin and obtained from formalin-fixed and paraffin-embedded tissue block.

Table 1: Individual data (Beha *et al*., 2012)

N°	AGE	BREED	OS – 730days*
1	9	mixed breed	
2	12	German shepherd	
3	7	Yorkshire terrier	D (120)
4	6	English setter	D (540)
5	10	Dobermann	
6	13	mixed breed	
7	11	Maremma shepherd	A
8	13	mixed breed	
9	11	mixed breed	
10	11	mixed breed	A
11	8	Dobermann	D (600)
12	11	mixed breed	
13	8	mixed breed	D (320)
14	10	German shepherd	A
15	11	mixed breed	D (450)
16	14	mixed breed	A
17	10	mixed breed	A
18	10	English setter	D (60)
19	10	mixed breed	
20	11	poodle	

*OS= Over-survival time after 730 days; D= dead; A= alive

Histological diagnosis and immunohistochemistry

Histological diagnosis was made according to the WHO classification system [6]. Seven consecutive 4µm-thick sections were cut from the paraffin blocks containing representative tumour samples and labeled by immunohistochemistry with the following antibodies: anti-ER, -PR, -c-erbB-2, -CK5/6, -CK14, -CK19, -p63. Data on the primary antibodies are summarized in Table 2.

Table 2: Primary antibodies, resources and dilutions used in immunohistochemistry (Beha *et al*., 2012)

ANTIBODY (−ANTI)	CLONE	MANUFACTURER	DILUTION
ER	B-10	Abcam, Cambridge, UK	1: 300
PR	PR4-12	Oncogene TM, Boston, MA, USA	1: 100
c-erbB-2	Polyclonal	Dako, Glostrup, Denmark	1: 250
Cytokeratins 5/6	D5/16B4	Zymed (South San Francisco, CA, USA)	1: 100
Cytokeratin 14	Ab-1 (LL002)	NeoMarkers (Fremont, CA, USA)	1: 300
Cytokeratin 19	BA17	Dako (Glostrup, Denmark)	1: 50
p63	4A4	Dako (Glostrup, Denmark)	1: 50

Sections were dewaxed in toluene and rehydrated. Endogenous peroxidase was blocked by immersion in H2O2 0.3% in methanol for 20 min. Sections were then rinsed in Tris Buffer and antigen was retrieved with citrate buffer (2.1 g citric acid monohydrate/liter distilled water), pH 6.0 (except for CK 5/6 which use EDTA, pH 8.0), and heating for two 5-min periods in a microwave oven at 750 W, followed by cooling at room temperature for 20 min. All antibodies were incubated with the tissue sections overnight at 4°C, and their binding was revealed by a commercial streptavidin-biotin-peroxidase technique (LSAB Kit, Dako, Amsterdam, The Netherlands). Diaminobenzidine (0.05% for 10 min at room temperature) was used as chromogen. Slides were counterstained with Papanicolaou's hematoxylin.

As a negative control, the primary antibody was replaced with an irrelevant, isotype-matched antibody to control for non-specific binding of the secondary antibody. As positive controls to assess the cross-reactivity with canine tissues and the specificity of the immunohistochemical stain, sections of normal canine uterus (for anti-ER and -PR antibodies), canine skin (for anti-CK5/6, -CK14, -CK19 and -p63 antibodies) were used following the same protocols. A

human poorly differentiated invasive ductal mammary carcinoma (kindly provided by P. Viacava, Department of Oncology, University of Pisa, Italy) known to react with c-erbB-2 antibody was used as positive control.

The staining result was classified semi-quantitatively with a dichotomous evaluation: positive or negative. The sample was considered positive:

• when presenting cytoplasmic stain in more than 1% of the invasive tumour cells for anti-CK-19, CK-5/6 and anti-CK14 antibodies [26];

• when presenting complete membranous stain in more than 10% of tumour cells for anti- c-erbB-2 antibody according to the Hercept-test [27];

• when presenting nuclear stain in more than 5% of tumour cells for anti-ER and anti-PR antibodies [28];

• when presenting nuclear stain in more than 10% of tumour cells for anti-p63 antibody [29].

Molecular taxonomy

The application of the panel allowed cases to be grouped into five molecular subtypes according to an algorithm by Sassi et al. [9] modified as follows:

• Luminal-A: ER+ and/or PR+, c-erbB-2–, regardless of CK5/6, CK14, p63 staining.

• Luminal-B: ER+ and/or PR+, c-erbB-2+, regardless of CK5/6, CK14, p63 staining.

• c-erbB-2 overexpressing: ER–, PR–, c-erbB-2+ regardless of CK5/6, CK14, p63 staining.

• Basal-like: ER–, PR–, c-erbB-2–, CK5/6+ and/or CK14+ and/or p63+.

• Normal-like: Negative to all markers.

Results

Diagnosis

Eight of the 20 primary tumour were classified as simple tubulopapillary carcinomas, eight as solid carcinomas, two as complex carcinomas and two as anaplastic carcinomas.

Immunohistochemistry and molecular phenotypes

Immunohistochemistry for ER, PR, c-erbB-2, CK5/6, CK14, p63 in the primary tumour and in the respective lymph node metastasis is summarized in Table 3. In each case the epithelial origin of cancer was confirmed by CK19 staining. Based on the applied algorithm, molecular phenotypes were obtained in the primary mammary tumour and in their lymph node metastases (Table 4). Four phenotypes (luminal A (Figure 1A-B, line 1-2-3), luminal B (Figure 2A-B, line 1-2-3-4), c-erbB-2 overexpressing (Figure 3A-B), basal-like (Figure 4A-B-C)) were diagnosed in primary tumour (eight (40 %), seven (35 %), two (10%), three (15%) respectively) and five (luminal A (Figure 1C-D-E, line 1), luminal B (Figure 2C-D-E, line 1), c-erbB-2 overexpressing (Figure 2C-D-E, line 2 and Figure 3c-d-e), basal-like (Figure 2c-d-e, line 3 and Figure 4c-d-e), normal-like (Figure 1c-d-e, line 3 and line 4 of Figure 2c-d-e)) in the lymph node metastases (five (25%), three (15%), four (20%), six (30%), two (10%) respectively).

Relationship between molecular phenotype in the primary mammary tumour and its related lymph node metastasis

Phenotypic concordance was found in 13 of the 20 cases (65%) (five luminal A (Figure 1, line 1), three luminal B (Figure 2, line 1), two c-erbB-2 overexpressing (Figure 3) and three basal-like (Figure 4)). Seven cases (35%) showed discordance with lymph node phenotypic profile differing from the primary tumour (two luminal A became basal-like (Figure 1, line 2), one luminal A became normal-like (Figure 1, line 3), two luminal B became c-erbB-2

overexpressing (Figure 2, line 2), one luminal B became basal-like (Figure 2, line 3), one luminal B became normal-like (Figure 2, line 4)) (Table 3). C-erbB-2 overexpressing and basal-like primary tumour were 100% concordant with the molecular phenotype of the respective lymph node metastases (Figure 3 and Figure 4). Luminal A and luminal B were concordant in 65.2% and 42.9% respectively of primary tumour for the same comparison.

Table 3: Summary of immunohistochemical staining

| SAMPLES ID | PRIMARY MAMMARY TUMOR | | | | | | LYMPH NODE METASTASIS | | | | | |
	ER*	PR*	c-erbB-2°	CK 14*	CK 5/6*	p63*	ER*	PR*	c-erbB-2°	CK 14*	CK 5/6*	p63*
1	1	1	1	1	1	0	0	1	1	1	1	0
2	0	1	1	1	1	0	0	1	1	1	1	0
3	1	0	1	1	1	1	0	0	0	0	0	0
4	0	0	1	1	1	0	0	0	1	1	1	0
5	0	1	3	1	1	1	0	1	3	1	1	0
6	1	0	1	1	1	1	0	0	1	1	1	1
7	1	1	1	1	1	1	1	1	0	1	1	0
8	0	1	1	1	1	0	0	0	1	1	1	0
9	1	1	3	1	1	1	0	0	3	1	1	0
10	1	1	3	1	1	1	1	1	3	1	1	0
11	0	0	1	1	0	0	0	0	1	1	0	0
12	1	0	2	1	1	1	0	0	1	1	1	0
13	1	0	2	1	1	1	0	0	1	0	0	0
14	1	1	1	1	1	0	1	0	1	1	1	0
15	0	0	2	1	1	0	0	0	3	1	1	0
16	1	0	1	1	1	0	1	0	1	0	1	0
17	0	1	2	1	1	0	0	1	2	1	1	0
18	0	0	2	1	1	1	0	0	2	0	0	0
19	0	0	1	1	1	0	0	0	1	1	1	0
20	0	1	2	1	1	1	0	0	2	1	1	1

*ER, PR, CK5/6, CK14, p63: 0= no immunoreactivity; 1= immunoreactivity. °c-erbB-2: 0= no immunoreactivity; 1= <10% positive cells; 2= 10 to 90% complete membranous immunostaining; 3= >90% complete membranous cell positivity

Table 4: Molecular phenotypes

SAMPLES ID	PHENOTYPE		PHENOTYPIC RELATIONSHIP
	PRIMARY TUMOR	LYMPH NODE METASTASIS	
1	Luminal A	Luminal A	Concordance
2	Luminal A	Luminal A	Concordance
3	Luminal A	Normal-like	Discordance
4	Basal-like	Basal-like	Concordance
5	Luminal B	Luminal B	Concordance
6	Luminal A	Basal-like	Discordance
7	Luminal A	Luminal A	Concordance
8	Luminal A	Basal-like	Discordance
9	Luminal B	c-erbB-2 overexpressing	Discordance
10	Luminal B	Luminal B	Concordance
11	Basal-like	Basal-like	Concordance
12	Luminal B	Basal-like	Discordance
13	Luminal B	Normal-like	Discordance
14	Luminal A	Luminal A	Concordance
15	c-erbB-2 overexpressing	c-erbB-2 overexpressing	Concordance
16	Luminal A	Luminal A	Concordance
17	Luminal B	Luminal B	Concordance
18	c-erbB-2 overexpressing	c-erbB-2 overexpressing	Concordance
19	Basal-like	Basal-like	Concordance
20	Luminal B	c-erbB-2 overexpressing	Discordance

Histological diagnosis in primary mammary tumour compared to molecular phenotypes and to concordance/discordance

In the primary tumour the luminal A phenotype displayed a different histological tumour pattern, i.e. simple tubulopapillary (one case) (Figure 1A-B, line 1), solid (six cases) (Figure 1A-B, line 2), anaplastic (one case) (Figure 1A-B, line 3). Luminal B phenotype displayed a

different histological tumour pattern, i.e. simple tubulopapillary (four cases) (Figure 2A-B, line 1 and 4), solid (two cases) (Figure 2A-B, line 2) and complex (one case) (Figure 2A-B, line 3). The c-erbB-2 overexpressing phenotype showed two patterns, i.e. tubulopapillary (one case), and anaplastic (one case) (Figure 3A-B). The basal-like phenotype displayed different histological tumour patterns, i.e. simple tubulopapillary (two cases) (Figure 4A-B-C) and complex (one case). No association was found between histological diagnosis and phenotype in the primary tumour (Pearson Chi-square, P=0.065; for statistics only the tubulopapillary and the solid carcinomas were considered because the other two types had only one case for phenotype).

Six cases with tubulopapillary pattern displayed concordance between the primary tumour and its related lymph node metastasis whereas the other two cases showed discordance. In the solid pattern concordance was found in five cases and discordance in three. In the anaplastic and complex patterns both concordance and discordance were present with one case for each type. Comparing the four histological patterns, no differences in the percentages of concordance/discordance were evident (Pearson Chi square P=0.857, for statistics only the tubulopapillary and the solid carcinomas were considered because the other two types had only one case for each phenotype).

Influence of the molecular phenotype and concordance/discordance on dog's survival rate

Table 1 reports the survival data of the 11 animals. Few data are available to group the subjects according to molecular phenotypes and concordance/discordance to perform survival analysis even though it appears that all five animals alive at 24 months post-surgery were concordant luminal A or luminal B cases. The other six dead animals bore primary tumour/lymph node concordant (2 basal-like and 2 c-erbB-2 overexpressing) or discordant (1

58

luminal A/normal like; 1 luminal B/normal-like) cases in which less differentiated molecular types were present in both sites or only in the lymph node compared to the live animals.

Discussion

Canine mammary carcinomas can become fatal due to the development of distant metastases. One of the most important prognostic factors in the diagnosis is the acknowledgment of metastases to the regional lymph node that represents an early step in metastatic spread [30]. Klopfleisch *et al.* [17] and Lu *et al.* [31] demonstrated that metastatic spread of canine mammary tumour to the lymph nodes is associated with a gene expression profile of increased cell cycle progression, altered cell differentiation and decreased growth factor signaling. Metastasis development is a complex process involving invasion, intravasation, survival in the bloodstream, extravasation and homing and proliferation at the site of metastasis [32]. Although some phenotypes showed greater aggressiveness and metastatic capability, only a selected subpopulation was able to metastatize in the multiple and heterogeneous tumour cell population. In this case the phenotype may have been transient and these selected cells have had an intrinsic program to transition to a phenotype enhancing their ability for heterotypic interaction and survival proliferation in distant organs [32] as Darwinian clonal evolution. Conversely, the metastatic process has also been described as a stochastic event, the primary tumour cells having equal metastatic capability, characterized by a phenotypic overlap between the primary tumour and its metastases [33,34]. Thus the identification of molecular phenotypes in primary tumour and metastases can provide predictive information on the most likely metastatic profile, not the condition in the primary tumour.

Sassi *et al.* [9] identified three phenotypes out of the four detected by Gama *et al.* [8], demonstrating that basal-like subtypes were associated with a better outcome than luminal A and luminal B tumour, in contrast with the findings of Gama *et al.* [8]. The prognostic role of

c-erbB-2 overexpression remains controversial despite what is known in human medicine. According to a study by Hsu *et al*. [35], the relationship between the clinical course and protein expression of c-erbB-2 in dogs with malignant mammary neoplasia indicated a greater survival rate in tumour overexpressing c-erbB-2 compared to those having nonoverexpressed levels of antigen. Certainly, c-erbB-2 plays an important role in carcinogenesis, but does not seem to be directly correlated with progression to malignancy [35]. In the present investigation it seems that luminal A or B concordance should be considered a positive prognostic factor, whereas concordance for the other molecular types or discordance should not, even if these results await confirmation in a larger number of cases and proper statistical analysis.

This study revealed four out of the five protein expression phenotypes of breast cancer in primary tumour (20 cases): luminal A (40%), luminal B (35%), c-erbB-2 overexpressing (10%) and basal-like (15%). The prevalence of luminal phenotypes (75%) over the others (25%) is in accordance with findings both in human [11,36,37] and veterinary [8;9] medicine.

Based on the present study and in agreement with Brunetti *et al*. [38], labeling for CK and p63 would only appear necessary when a tumour is negative for ER, PR and c-erbB-2. With regard to luminal A and B phenotypes, the expression profiles of ER and PR are essential to decide on the application of endocrine therapy [39] in breast cancer and canine mammary neoplasia, and also seem to play a minor role in predicting tumour biological behaviour [40,41]. Wu *et al*.'s study [15] in breast cancer confirmed the observation that ER and/or PR could be lost when carcinomas metastasizes, thereby resisting endocrine therapy. The present study shows almost overlapping results, losing hormone receptors by moving from luminal A to basal-like (2 cases) and/or to normal-like (1 case) phenotypes and from

luminal B to c-erbB-2 overexpressing (2 case) and/or to basal-like (1 case), and/or to normal like (1case), confirming that the gene expression profile in canine mammary tumour may prove a helpful tool in clinical practice. Chang *et al*. [42] indicated that the ER or PR expression in dogs was associated with tumour size, clinical stage, and lymph node metastasis or distant metastases. Dogs with malignant mammary neoplasia and expression of both ER and PR had a longer survival rate than dogs with malignant mammary tumour that were ER positive but PR negative. This latest information on PR suggested that the receptor was a better outcome predictor than ER status alone and that its positive or negative expression could serve as a prognostic factor, especially in dogs with malignant neoplasia with ER expressed [42]. The present results disclosed a high prevalence of hormone receptor expression in the primary tumour, whose positivity was ensured by reactivity to least one of the two markers (ER and/or PR) (Table 3). The latest results indicate that there are grounds for the use of anti-hormone therapy in dogs, administering molecules other than those hitherto used in veterinary medicine (tamoxifen) as their side-effects are already well-known [24]. A similar analysis in lymph node showed a net loss of hormone receptor expression, namely ER. ER loss is a known adverse prognostic factor [42], and therefore its lack of expression in metastases is indicative. In this study, only five out of the 20 cases showed positivity to both ER and PR in the primary tumour, with persistent positivity in the lymph node metastasis in only two cases. The remaining three cases showed loss of one or both hormone receptor staining in the lymph node metastasis: loss of ER (case n°1), loss of PR (case n° 14) and concomitant loss of ER and PR (case n° 9). According to the literature, these cases should have a poor prognosis, justified by our phenotypes, but with maintained luminality in the first two cases, and a shift to c-erbB-2 overexpression in the third meaning a even worse evolution by the complete loss of ER and PR.

Interestingly, cases n°3 and n°13 (Table 3) in which in lymph node metastasis occurred, lost

all the markers expressed in the primary tumour, likely due to the selection of a significantly aggressive cell subpopulation. The normal-like or multiple markers negative (MMN) subtype tumour have been shown to be negative for basal markers, such as CK5/6 and CK14 in our cases, and negative for other molecular markers. The majority of normal-like subtype tumour express CK8/18, CK19 (used in this study), with an absence of CK5/6 suggesting that these cells were most probably derived from a luminal gland cell [43]. The normal-like subtype is also included in the triple negative breast cancer group (TNBC) characterized by an aggressive clinical course, poor survival rate and, unlike the overexpressing hormone receptors or c-erbB-2-overexpressing tumour, is not amenable to hormone therapy or c-erbB-2-directed agents [44]. Although no correlation has been found between histological type and phenotype, the normal-like cases represent an exception.

The present study identified five phenotypes in the lymph node metastases: luminal A (25%), luminal B (15%), c-erbB-2 overexpressing (20%), basal-like (30%) and normal-like (10%). The novel aspect of this study is the evaluation of the lymph node metastasis phenotypes and their correlation with the primary tumour, never hitherto applied to canine species. The relationship between the primary tumour and metastatic phenotype is defined by a concordance in 65% of cases and a discordance in the remaining 35%, suggesting the two main metastatic capability theories coexist. All seven discordant cases showed a progressive behavior, according to the prognostic value of molecular phenotypes reported by Gama *et al*. [8], suggesting phenotypic evolution with a worse prognosis from the primary tumour to lymph node metastasis.
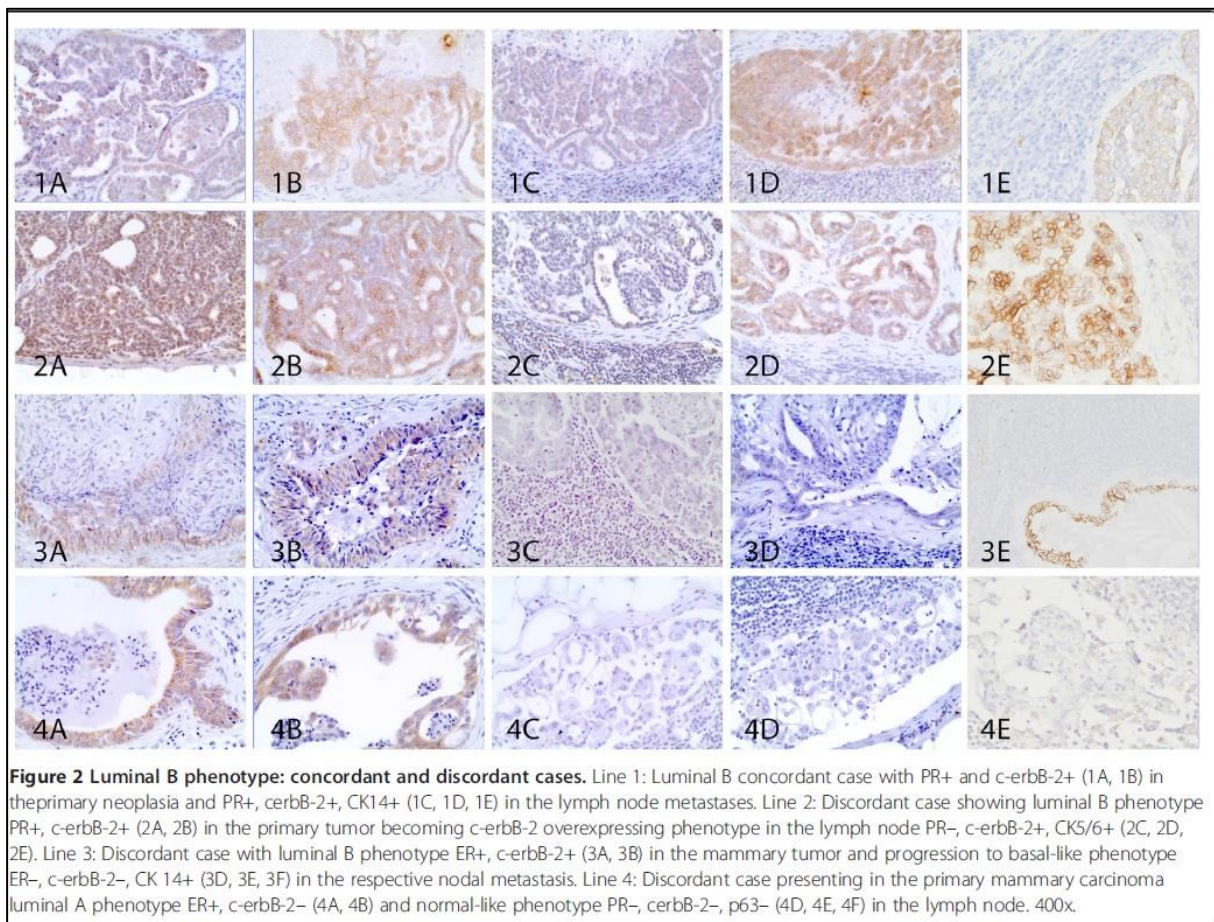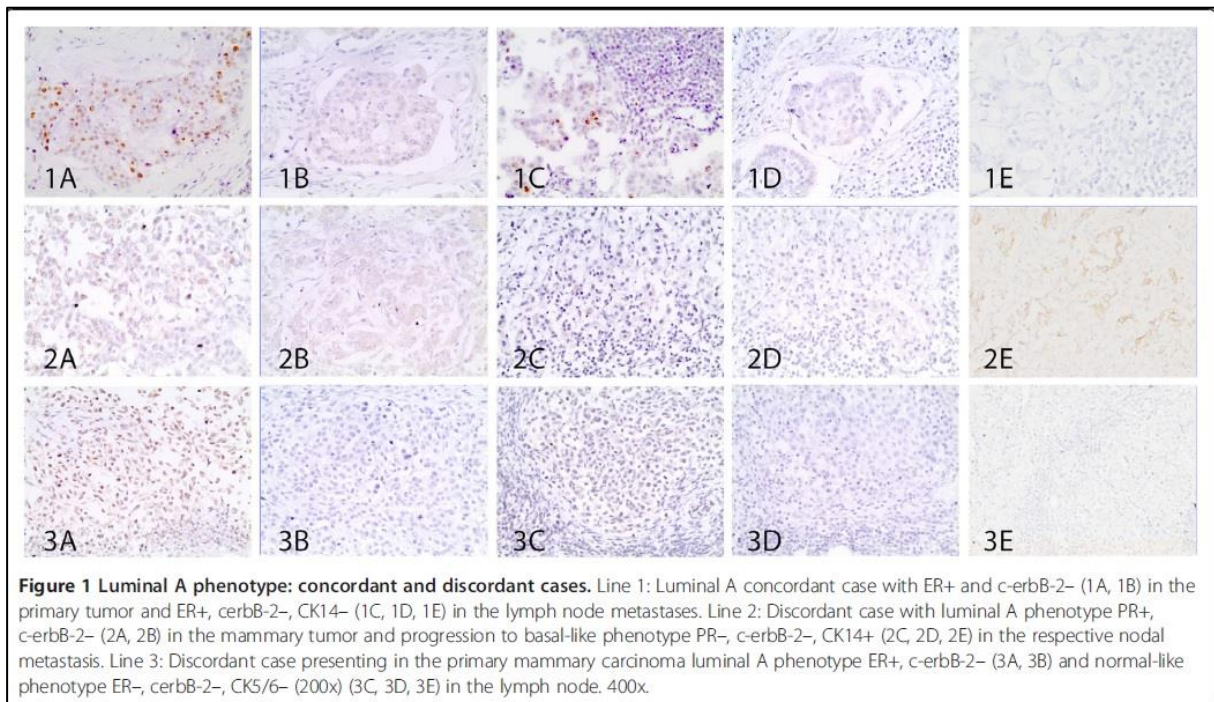
Conclusions

Molecular phenotype classification is a new model urgently needed in veterinary medicine. This model will fill current gaps regarding prognosis and a targeted therapeutic approach,

since the primary tumour phenotype does not always overlap with that of its metastasis. According to the present findings, the primary tumour phenotype assumes a predictive therapeutic role only in concordant cases, meaning that there should be a concomitant evaluation of both the primary tumour and its lymph node metastasis. Treatment planning based only on the primary tumour phenotype can lead to therapeutic failures if the lymph node metastatic phenotype differs from that of the primary tumour.
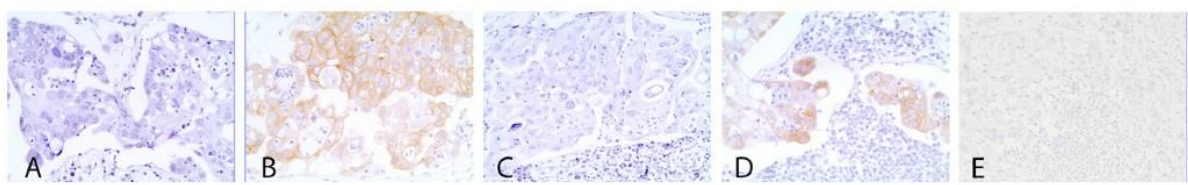
Figures

Figure 1 Luminal A phenotype: concordant and discordant cases. Line 1: Luminal A concordant case with ER+ and c-erbB-2– (1A, 1B) in the primary tumor and ER+, cerbB-2–, CK14– (1C, 1D, 1E) in the lymph node metastases. Line 2: Discordant case with luminal A phenotype PR+, c-erbB-2– (2A, 2B) in the mammary tumor and progression to basal-like phenotype PR–, c-erbB-2–, CK14+ (2C, 2D, 2E) in the respective nodal metastasis. Line 3: Discordant case presenting in the primary mammary carcinoma luminal A phenotype ER+, c-erbB-2– (3A, 3B) and normal-like phenotype ER–, cerbB-2–, CK5/6– (200x) (3C, 3D, 3E) in the lymph node. 400x.

Figure 2 Luminal B phenotype: concordant and discordant cases. Line 1: Luminal B concordant case with PR+ and c-erbB-2+ (1A, 1B) in theprimary neoplasia and PR+, cerbB-2+, CK14+ (1C, 1D, 1E) in the lymph node metastases. Line 2: Discordant case showing luminal B phenotype PR+, c-erbB-2+ (2A, 2B) in the primary tumor becoming c-erbB-2 overexpressing phenotype in the lymph node PR–, c-erbB-2+, CK5/6+ (2C, 2D, 2E). Line 3: Discordant case with luminal B phenotype ER+, c-erbB-2+ (3A, 3B) in the mammary tumor and progression to basal-like phenotype ER–, c-erbB-2–, CK 14+ (3D, 3E, 3F) in the respective nodal metastasis. Line 4: Discordant case presenting in the primary mammary carcinoma luminal A phenotype ER+, c-erbB-2– (4A, 4B) and normal-like phenotype PR–, cerbB-2–, p63– (4D, 4E, 4F) in the lymph node. 400x.

Figure 3 C-erbB-2 overexpressing phenotype: concordant case. PR–, c-erbB-2+ (1A, 1B) in mammary tumor and PR–, c-erbB-2+, p63– (200x) (2C, 2D, 2E) in the respective lymph node metastasis. 400x.
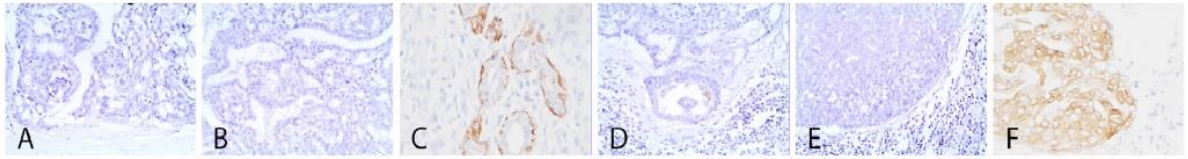

Figure 4 Basal-like phenotype: concordant case. ER–, c-erbB-2–, CK5/6+ (1A, 1B, 1C) in mammary neoplasia and PR–, c-erbB-2–, CK 14+ (2C, 2D, 2E) in the lymph node metastasis. 400x.

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Publications and Proceedings

1. Beha G., Brunetti B., Asproni P, Muscatello L.V., Millanta F., Poli A., Sarli G., Benazzi C. Molecular portrait-based correlation between primary canine mammary tumour and its lymph node metastasis: possible prognostic-predictive models and/or stronghold for specific treatments? BMC Veterinary Research 2012, 12;8:219

2. Beha G., Brunetti B., Asproni P., Muscatello L. V., Millanta F., Poli A., Sarli G., Benazzi C. Molecular portrait-based correlation between primary canine mammary tumour and its lymph node metastasis: possible prognostic-predictive models and/or stronghold for specific treatments? (Oral Presentation). Proceedings of the joint meeting of ESVP-ECVP, Leon, 5-8 September 2012, p. 62

3. **Beha G**., Brunetti B., Asproni P., Muscatello L.V., Millanta F., Poli A., Sarli, G. Benazzi. C. Correlation, Based on the Protein Expression Profile, Between Primary Canine Mammary Tumours and Their Lymph Node Metastases. Journal of Comparative Pathology, 01/2013; 148(1):48.

2. MOLECULAR PHENOTYPE IN MAMMARY TUMOURS OF QUEENS: CORRELATION BETWEEN PRIMARY TUMOUR AND LYMPH NODE METASTASIS

NEOPLASTIC DISEASE

Molecular Phenotype in Mammary Tumours of Queens: Correlation between Primary Tumour and Lymph Node Metastasis

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Introduction

The heterogeneity of breast cancer is proverbial and raises the need for a revision of the WHO classification, which is purely morphological (Eusebi, 2010). Sorlie *et al*. (2001) laid the foundations for a new taxonomy demonstrating that changes in patterns of gene expression, analyzed by cDNA microarray techniques and hierarchical clustering, allow a "molecular portrait" to be defined for each tumour. The final goal of this system is to classify the breast cancers into subtypes, Luminal-A, Luminal-B, c-erbB-2 over-expressing, basal-like and Normal-like, based on the differences between these patterns (Sorlie *et al*., 2001). The existence of four different subtypes of breast cancer was confirmed by protein expression patterns assessed by IHC on tissue microarray (TMA), an efficient and reliable platform for sub-classifying breast cancers into relevant subtypes, using a limited number of markers

(Matos *et al*., 2005). So far, this molecular classification has been applied in veterinary medicine only in canine (mammary).

The usefulness of molecular subtypes is their predictive capability for prognosis and targeted therapy (Peppercorn *et al*., 2008). The formulation of a molecular-based taxonomy and its application to feline clinical practice is necessary for a multimodal therapeutic approach and to increase the survival rate.

Aim of the present study was: 1) to define the molecular phenotype of feline mammary carcinomas and their lymph node metastases according to a modified algorithm by Sassi *et al*. (2010); 2) to demonstrate the concordance or discordance of the molecular profile between the primary tumour and lymph node metastasis.

Materials and Methods

Samples

Specimens of mammary carcinomas from 21 female cats were collected from the data base of the Pathology Service of the Department of Veterinary Medical Science of Bologna University and from the Department of Animal Pathology of Pisa University. Each case consisted in primary mammary tumour and its related lymph node metastasis. No cases displayed systemic metastases.   Samples were available as haematoxylin and eosin stained sections, obtained from formalin-fixed and paraffin wax-embedded tissue block.

Histological diagnosis and immunohistochemistry

Histological diagnosis was achieved according to the WHO classification system (Misdorp et al., 1999). Seven consecutive 4 μm thick sections were cut from the paraffin wax blocks containing representative tumour samples and labelled by immunohistochemistry with the

following antibodies: anti-ER, -PR, -c-erbB-2, -CK5/6, -CK14, -CK19 and -p63. Data on the primary antibodies are summarized in Table 1.

Table 1: Primary antibodies, resources and dilutions used in immunohistochemistry

ANTIBODY (-ANTI)	CLONE	MANUFACTURER	DILUITION
ER	6F11	Novocastra Lab Ltd., Newcastle upon Tyne, UK	1: 40
PR	PR88	Biogenex, San Ramon, CA, USA	1: 40
c-erbB-2	Policlonal	Dako, Glostrup, Denmark	1: 250
Cytokeratins 5/6	D5/16B4	Zymed (South San Francisco, Ca)	1: 100
Cytokeratin 14	Ab-1 (LL002)	NeoMarkers (Fremont, Ca)	1: 300
Cytokeratin 19	BA17	Dako (Glostrup, Denmark)	1: 50
p63	4A4	Dako (Glostrup, Denmark)	1: 50

Sections were dewaxed in toluene and rehydrated. Endogenous peroxidase was blocked by immersion in 0.3% hydrogen peroxide for 20 min. Sections were then rinsed in Tris Buffer and antigen was retrieved with citrate buffer (2.1 g citric acid monohydrate/litre distilled water), pH 6.0 (except for CK 5/6 which use EDTA, pH 8.0), and heating for two 5-min periods in a microwave oven at 750 W, followed by cooling at room temperature for 20 min. All antibodies were incubated with the tissue sections overnight at 4°C, and their binding was revealed by a commercial streptavidin-biotin-peroxidase technique (LSAB Kit, Dako, Amsterdam, The Netherlands). Diaminobenzidine (0.05% for 10 min at room temperature) was used as chromogen. Slides were counterstained with Papanicolaou's haematoxylin.

As negative control, the primary antibody was replaced with an irrelevant, isotype-matched antibody to control for non-specific binding of the secondary antibody. As positive controls to assess the cross-reactivity with feline tissues and the specificity of the immunohistochemical

stain, sections of normal feline uterus (for anti-ER and -PR antibodies) and feline skin (for anti-CK5/6, CK14, -CK19 and -p63 antibodies) were used following the same protocols. A human poorly differentiated invasive ductal mammary carcinoma (kindly provided by P. Viacava, Department of Oncology, University of Pisa, Italy) known to react with c-erbB-2 antibody was used as positive control.

The staining result was classified semi-quantitatively with a dichotomous evaluation: positive or negative. The sample was considered positive when presenting:

• cytoplasmic stain in more than 1% of the invasive tumour cells for anti-CK-5/6 and anti-CK14 antibodies (Kim *et al*., 2006);

• complete membranous stain in more than 10% of tumour cells for anti-c-erbB-2 antibody (Millanta *et al*., 2005b);

• nuclear stain in more than 5% of tumour cells for anti-ER and anti-PR antibodies (Millanta *et al*., 2005a);

• nuclear stain in more than 10% of tumour cells for anti-p63 antibody (Ramalho *et al*. 2006).

Molecular taxonomy

The application of the panel grouped cases into five molecular subtypes according to an algorithm modified by Sassi *et al.* 2010 as follows:

• Luminal-A: ER+ and/or PR+, c-erbB-2-, regardless of CK5/6, CK14, p63 staining.

• Luminal-B: ER+ and/or PR+, c-erbB-2+, regardless of CK5/6, CK14, p63 staining.

• c-erbB-2-overexpressing: ER-, PR-, c-erbB-2+ regardless of CK5/6, CK14, p63 staining.

• Basal-like: ER-, PR-, c-erbB-2- , CK5/6+ and/or CK14+ and/or p63 staining.

• Normal-like: negative to all markers.

Results

Diagnosis

Six of the 21 primary tumours were classified histologically as solid carcinomas and 15 as simple tubulo-papillary carcinomas.

Immunohistochemistry

The immunohistochemistry for ER, PR, c-erbB-2, CK5/6, CK14, p63 in the primary tumour and respective lymph node metastasis is summarized in Table 2. In each case the epithelial origin of cancer was confirmed by CK19 staining. Hormonal receptor positivity (reactivity to ER and/or PR) was found in 11 out of 21 cases in the primary tumour, two ER and nine PR positive respectively (Table 2). The typical nuclear staining of ER and PR decreased substantially in the lymph node metastasis compared with the primary tumour (reactivity in only two out of 21 cases). C-erbB-2 over-expression was frequently observed both in the primary tumour (19 positive cases) and in the lymph node metastases (18 positive cases).

As reported in Table 2, CK5/6 expression was less frequent than CK14 expression both in primary tumours (11/21 and 19/21, respectively) and in their lymph node metastases (9/21 and 14/21, respectively). P63 was poorly expressed in primary tumours (four out of 21 samples) and especially in metastases (one out of 21).

Molecular phenotypes

Based on the algorithm applied, molecular phenotypes were obtained in the primary mammary tumour and in their lymph nodes metastases (Table 3). Only three phenotypes (Luminal B (Fig. 1), c-erbB-2 over-expressing (Fig. 2), Basal (Fig. 3)) were diagnosed both

in primary tumours (11 (52.4%), eight (38.1%) and two (9.5%), respectively) and in lymph

node metastases (two (9.5%), 16 (76.2%) and three (14.3%), respectively).

Table 2: Summary of immunohistochemical staining

SAMPLES ID	PRIMARY MAMMARY TUMOUR						LYMPH NODE METASTASIS					
	ER	PR	c-erbB-2	CK 14	CK 5/6	p63	ER	PR	c-erbB-2	CK 14	CK 5/6	p63
1	0	1	3	1	1	0	0	1	3	1	1	0
2	0	0	2	1	0	0	0	0	2	1	0	0
3	1	0	2	1	0	1	0	0	2	1	0	0
4	0	1	1	1	0	0	0	0	1	0	0	0
5	0	1	2	1	0	0	0	0	2	1	0	0
6	1	0	3	1	1	0	0	0	2	0	0	0
7	0	0	0	1	0	0	0	0	0	1	0	0
8	0	0	1	1	1	0	0	0	1	0	0	0
9	0	0	2	1	1	0	0	0	2	1	1	0
10	0	0	2	1	1	1	0	0	1	1	0	0
11	0	0	3	1	0	0	0	0	3	1	1	0
12	0	1	1	1	1	0	0	0	0	1	1	0
13	0	0	3	1	1	0	0	0	3	1	1	0
14	0	1	3	1	0	0	0	0	3	0	0	0
15	0	1	3	1	1	1	0	0	3	1	1	0
16	0	0	2	1	0	0	0	0	2	1	0	0
17	0	1	2	0	1	0	0	0	2	0	1	0
18	0	1	2	1	1	1	0	0	2	1	1	1
19	0	0	2	0	0	0	0	0	2	0	0	0
20	0	1	2	1	1	0	0	1	1	1	1	0
21	0	0	0	1	0	0	0	0	0	1	0	0

0= 0%; 1= <25%; 2 <50%; 3 >50%

Comparison between histological diagnosis and phenotype in primary mammary tumour

The tubulopapillary pattern displayed three different phenotypes, i.e. Luminal B (seven cases), c-erbB-2 over-expressing (six cases) and Basal-like (two cases). The solid pattern was found in two phenotypes, namely Luminal B (five cases) and c-erbB-2 over-expressing (one case). No association was found between histological diagnosis and phenotype in primary tumour (Yates corrected Chi-square, P=0.46).

Relationship between molecular phenotype in the primary mammary tumour and its related lymph node metastasis

Phenotypic concordance was found in 12 of the 21 cases (57.14%) (eight c-erbB-2 over-expressing, two Basal and two Luminal B). The phenotypic profile of primary tumours showed discordance from regional lymph node metastases in nine cases (42.86%) (eight Luminal B became c-erbB-2 over-expressing and one Luminal B became Basal-like in the lymph node metastasis) (Table 3).

Relationship between histological diagnosis and concordance/discordance

The same molecular phenotype (concordance) was found in the primary tumour and its lymph node metastasis in ten tubulo-papillary carcinomas, whereas a difference (discordance) between the two sites was found in the other five. As for the solid pattern concordance was found in two cases and discordance in four. No difference in the percentage of concordant and discordant cases emerged comparing the two histological patterns mentioned above (Yates corrected Chi-square, P=0.36).

Discussion

To the best of our knowledge, no literature reports have addressed the immunophenotyping of feline mammary carcinomas. Only single markers such as c-erbB-2 over-expression and steroid receptors have been used to date (Millanta *et al.*, 2005b; Rasotto *et al.*, 2011). The present study analysed markers (ER, PR, c-erbB-2, CK5/6, CK14, p63) both singly and combined in the molecular phenotypes to detect possible changes from the primary tumour to its regional lymph node metastasis. Immunophenotyping is essentially based on the presence/absence of hormonal receptors (Fig. 1) and c-erbB-2 (Fig. 2), and basal markers (CK5/6, CK14 and p63) are the key for the phenotypic definition of negative hormone receptors (basal (Fig. 3) and normal-like) and c-erbB-2 tumours. Based on the present study, only if ER, PR and c-erbB-2 are negative is CK necessary (Fig. 3), otherwise it is useless and expensive. Gama et al. demonstrated in dogs that basal subtypes rarely express just one basal marker, but they frequently express several concomitant markers (Gama *et al.*, 2008). The present study exhibits some overlap with Gama's results regarding basal phenotypes, and suggests a more undifferentiated profile. Lymph node metastases did not show any immunostaining to the primary tumour markers. No protein absent in the tumour was expressed in its metastasis.

Given the well-known malignancy of feline mammary carcinomas (Misdorp, 1999) and the high frequency of c-erbB-2 over-expression, particularly in metastases, in the present study, it can be assumed that the c-erbB-2 over-expressing phenotype (Fig. 2) also leads to a worse prognosis in the cat as in human breast cancer (Sorlie *et al.*, 2001). Planning an anti-c-erbB-2 targeted therapy in queens bearing mammary carcinoma would therefore have a specific therapeutic value to counteract metastases.

In human medicine Toft *et al*. (2011) described the Basal-like phenotype as a distinctive molecular subtype with a basal epithelial gene signature and an aggressive clinical course characterized by early relapses and poor survival. They also found a prevalence of Basal-like breast cancer ranging from 12.3 to 36.7 of breast cancer cases (Toft *et al*., 2011). This range is similar to the percentage frequency found in this study on mammary tumours in queens, but the absence of outcome precludes any comment on the similarity between human and feline Basal-like phenotypes.

The analysis of hormone receptors status disclosed a low expression of these markers both in the primary tumours and more markedly in their metastases. Indeed, normal mammary tissue and benign mammary tumours are mostly positive for ER and PR with respect to carcinomas, which are more often ER negative or positive in only few cases (Misdorp, 2002). High negative rates of ER seem to be a characteristic feature of feline mammary carcinomas compared with human and canine tumours, suggesting a lack of oestrogen dependence (Millanta *et al*., 2005a). The high number of ER/PR negative cases in cat mammary carcinomas reduces the frequency of the tumour classified as Luminal type by the applied tumour classification system: in this study Luminal A carcinomas were lacking and the frequency of Luminal B (Fig. 1) lesions was lower than c-erbB-2 over-expressing tumours (Fig. 2). The results suggest an association between a down-regulation of p63 expression and progression of the tumour mirroring breast ductal carcinoma (Wang *et al.,* 2002; Lindsay *et al.*, 2011). C-erbB-2 (Fig. 2) in this study was always over-expressed with a high frequency in the primary site as well as in the metastases associated with ER/PR expression (Luminal B) or not (c-erbB-2 over-expressing). Therefore anti-hormonal therapy alone is not indicated in queens bearing mammary carcinomas, even in cases with ER/PR expression, but should be associated with a target anti-c-erbB-2 therapy.

It has been debated for decades how cancer cells acquire metastatic capability. It is unclear whether metastases are derived from a distinct tumour cell subpopulation with higher metastatic potential within the primary site, or whether they originate from a random fraction of tumour cells (Weigelt *et al*., 2003). Our comparison between the phenotypes of a primary tumour and its lymph node metastasis displayed concordance in 57.1% cases and discordance in 42.9%, with a progressive loss of marker immunoreactivity in the lymph node metastasis with respect to the primary tumour. Human and canine Luminal A tumours are associated with higher survival rates and Basal-like and c-erbB-2-overexpressing subtypes are related to lower survival rates and more aggressive clinical behavior (Matos *et al*., 2005; Gama *et al*., 2008). Hence, the progressive phenotypic discordance found in this study may signify a worse prognosis with worsening of the phenotype passing from the primary tumour to the lymph node metastasis. Phenotypic instability could be due to a tumoural cell sub-population that acquires metastatic ability during carcinogenesis. Conversely, phenotypic concordance of the primary tumour and metastasis could result from an intrinsic and early metastatic capability of the dominant phenotype in the primary tumour. Weigelt *et al*. (2005) reported that the ability to metastasize to distant sites is an early and inherent genetic property of primary breast cancer, and that gene expression profiles of primary breast tumours were maintained in their distant metastases (Weigelt *et al*. 2005).

 An overall concordance has been proven in the immunostaining of the single markers between primary tumours and metastasis, especially for c-erbB-2 over-expressing lesions, indicating that the cat may develop the selection of an early clone with worsening prognostic features rather than a malignant tumour progression. The discordance between the single markers, also reported in human medicine (Aitken *et al*., 2010), led to therapeutic failure when the therapy was only based on the primary tumour phenotype. Histological type is not

associated with the molecular phenotype, since several phenotypes are related to each histological type.

Conclusions

From a molecular standpoint, the taxonomic approach represents a powerful tool for the biological analysis of mammary tumours. The heterogeneity of the tumour is well-known, therefore taxonomy based on these molecular differences is the key tool to formulate an appropriate therapy. As for the difference between the primary tumour and the regional lymph node metastasis, the primary tumour phenotype was not strictly predictive of the metastic phenotype in a high percentage of cases (43%) in this study. Both tumour sites (primary tumours and regional lymph node metastases) should be evaluated for accurate identification of the tumour profile (or profiles) and appropriate therapy designed to target both primary tumours and metastases in case of varying phenotypes.

Figures
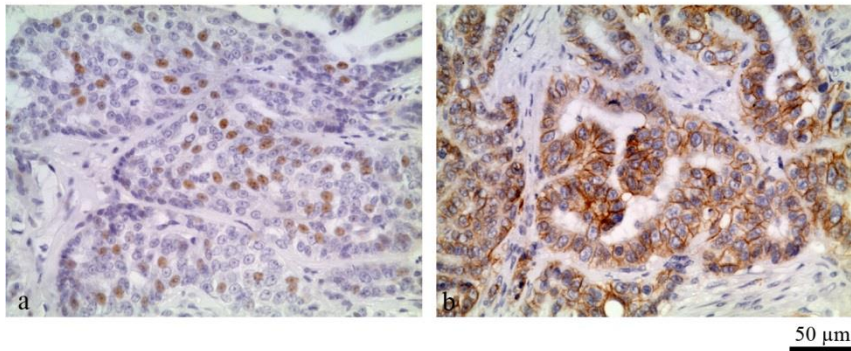
Fig. 1: Case 3. Luminal B phenotype: a ER staining, b c-erbB-2 staining. To be classified as luminal B phenotype, a neoplasm requires positivity to ER and/or PR and to c-erbB-2, while the results of the other antibodies (CK5/6 +/- , CK14+/-, p63+/-) are irrelevant. 400x.
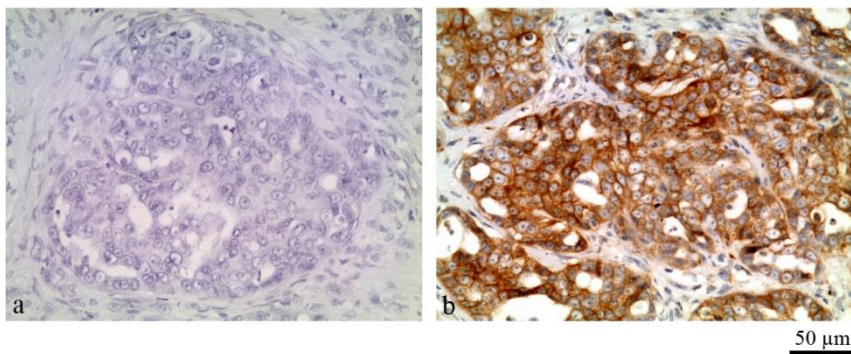

Fig. 2: Case 8. C-erbB -2-overexpressing phenotype: a ER staining, b c-erbB-2 staining. For the diagnosis of the c-erbB-2 phenotype, besides positivity to c-erbB-2 antibody, negativity to ER and PR is necessary, whereas the outcomes of the other antibodies (CK5/6 +/- , CK14+/-, p63+/-) are irrelevant. 400x
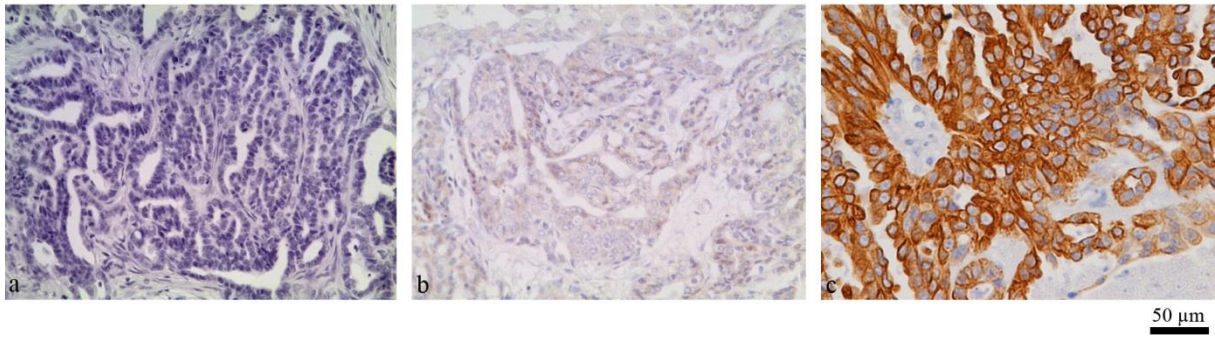
Fig. 3: Case 21. Basal-like phenotypes: a PR staining, b c-erbB-2 staining, c CK14 staining. Negativity to c-erbB-2, ER and PR and positivity to either CK (CK14 in the photo) or p63 is indicative of a basal phenotype. 400x

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1. Brunetti B., Asproni P., **Beha G**., Muscatello L.V., Millanta F., Poli A., Benazzi C., Sarli G. Molecular Phenotype in Mammary Tumours of Queens: Correlation between Primary Tumour and Lymph Node Metastasis. J. Comp. Path. 2013, 148(2-3):206-13).

2. Brunetti B., Asproni P., **Beha G**., Muscatello L.V, Millanta F., Poli A, Benazzi C., Sarli G. Fenotipo molecolare dei tumouri mammari della gatta: correlazione tra tumoure primario e relativa metastasi linfonodale. Atti AIPVET, Perugia 25-26 May 2012, p. 7-12.

3. MOLECULAR PHENOTYPE OF PRIMARY MAMMARY TUMOURS AND DISTANT METASTASES IN FEMALE DOGS AND CATS

NEOPLASTIC DISEASE

Molecular Phenotype of Primary Mammary Tumours and Distant Metastases in Female Dogs and Cats

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Introduction

In terms of prognosis and treatment approach, it is already well explained in the general section that the different phenotypes are associated with different clinical outcomes (Carey, 2011; Sorlie *et al*., 2003).

In the light of published findings, the metastatic process, i.e. the tumour's ability to spread from its primary location to distant parts of the body (Banfalvi, 2012), has become one of the most urgent, important and difficult issues to approach in human (Tarin, 2008) and animal cancer medicine. Among the possible sites of metastases, human (Tavassoli and Devilee, 2003), canine (Klopfleisch *et al*., 2010) and feline (Ginn *et al*., 2007) mammary tumours are known to spread to the regional lymph nodes, lungs, pleura, liver, adrenal glands, brain, kidneys and bone (Fountzilas *et al*., 2012; Kennecke *et al.,* 2010). In human medicine, investigations conducted to associate breast tumour molecular subtypes with the site of

metastatic disease have reported conflicting results (Arnedos *et al.*, 2012; Fountzilas *et al*., 2012; Kennecke *et al.,* 2010). Some studies found a low risk of brain metastases with luminal A and B disease, but a high risk for pleural relapse (Smid *et al*., 2008). Basal-like and normal-like tumours had a distinctive pattern of relapse, with higher frequencies of lung, brain, liver, locoregional and distant lymph node metastasis (Arnedos *et al*., 2012; Fountzilas *et al*., 2012; Kennecke *et al.,* 2010) compared to the frequency of c-erbB-2 overexpressing phenotype. Other studies showed that locoregional relapse (Lester, 2010), and distant metastases in brain (Blows *et al*., 2010), lung, liver and soft tissues (Aleskandarany *et al*.; 2012; Fountzilas *et al*., 2012) were more common in c-erbB-2 overexpressing tumours compared to basal and normal-like phenotypes. Bone metastases also presented a higher frequency with luminal B disease (Fountzilas *et al*., 2012; Kennecke *et al.,* 2010).

Few veterinary studies have considered the molecular phenotype of lymph node metastases in comparison with the primary mammary tumour phenotype (Beha *et al*., 2012; Brunetti *et al*., 2013). The phenotype of feline mammary carcinomas and their lymph node metastases was recently determined (Brunetti *et al*., 2013), and the same comparison made for canine mammary tumours (Beha *et al*., 2012). No data are yet available on the molecular phenotypes of distant metastases and their correlation with the primary mammary tumour and its related lymph node metastasis.

Therefore, the present study aimed to evaluate the molecular trend of cancer from its primary location to metastatic sites in three cats and two dogs with mammary tumours.

Materials and Methods

Samples

Tissue samples of mammary carcinomas from two female dogs and three female cats were collected from the database of the Pathology Service of the Department of Veterinary Medical Science, University of Bologna, and from the Department of Veterinary Sciences, University of Pisa. All the animals underwent autopsy.

Cases were selected based on both the primary mammary neoplasm and histological grade III (grade III: invasive carcinoma with distant metastases) according to a previous study (Gilbertson *et al.*, 1983). Samples were available as sections stained with hematoxylin and eosin and obtained from formalin-fixed and paraffin-embedded tissue blocks.

Histological diagnosis and immunohistochemistry

Histological diagnosis was made according to the WHO classification system (Misdorp *et al*., 1999). Six consecutive 4-μm thick sections were cut from the paraffin blocks containing representative tumour samples and three of them were labeled by immunohistochemistry with the following antibodies: anti-ER, -PR, -c-erbB-2. According to a previous study (Brunetti *et al*., 2013), samples presenting negative staining to the above antibodies were labeled by immunohistochemistry with anti-CK5/6, -CK14, and -p63 antibodies. Data on the primary antibodies are summarized in Table 1.

Sections were dewaxed in toluene and rehydrated. Endogenous peroxidase was blocked by immersion in H_2O_2 0.3% in methanol for 20 min. Sections were then rinsed in Tris buffer and antigen was retrieved with citrate buffer (2.1 g citric acid monohydrate/liter distilled water), pH 6.0 (except for CK5/6 which used EDTA, pH 8.0), and heated for two 5-min periods in a microwave oven at 750 W, followed by cooling at room temperature for 20 minutes.

All antibodies were incubated with the tissue sections overnight at 4°C, and their binding was revealed by a commercial streptavidin-biotin-peroxidase technique (LSAB Kit, Dako, Amsterdam, The Netherlands). Diaminobenzidine (0.05% for 10 minutes at room

temperature) was used as chromogen. Slides were counterstained with Papanicolaou's hematoxylin. As a negative control, the primary antibody was replaced with an irrelevant, isotype-matched antibody to control for non-specific binding of the secondary antibody. As positive controls to assess the cross-reactivity with canine and feline tissues and the specificity of the immunohistochemical stain, sections of normal canine and feline uterus (for anti-ER and –PR antibodies), canine and feline skin (for anti-CK5/6, -CK14, and -p63 antibodies) were used following the same protocols. A human poorly differentiated invasive ductal mammary carcinoma (kindly provided by P. Viacava, Department of Oncology, University of Pisa, Italy) known to react with c-erbB-2 antibody was used as positive control.

Table 1: Immunohistochemical panel of antibodies

ANTIBODY (-ANTI)	CLONE	MANUFACTURER	DILUTION
ER (dog)	B-10	Abcam, Cambridge, UK	1: 300
ER (cat)	6F11	Novocastra Laboratories Ltd., Newcastle upon Tyne, UK	1:40
PR (dog)	PR4-12	Oncogene TM, Boston, MA, USA	1: 100
PR (cat)	PR88	Biogenex, San Ramon, CA, USA	1:40
c-erbB2	Polyclonal	Dako, Glostrup, Denmark	1: 250
Cytokeratins 5/6	D5/16B4	Zymed (South San Francisco, CA, USA)	1: 100
Cytokeratin 14	Ab-1 (LL002)	NeoMarkers (Fremont, CA, USA)	1: 300
Cytokeratin 19	BA17	Dako (Glostrup, Denmark)	1: 50
p63	4A4	Dako (Glostrup, Denmark)	1: 50

The staining result was classified semi-quantitatively with a dichotomous evaluation: positive or negative. The sample was considered positive when presenting:

• cytoplasmic stain in more than 1% of the invasive tumour cells for anti- CK5/6 and anti-CK14 antibodies (Kim *et al*., 2006);

• complete membranous stain in more than 10% of tumour cells for anti- c-erbB-2 antibody according to the Hercept-test (Millanta *et al*., 2005b);

• nuclear stain in more than 5% of tumour cells for anti-ER and anti-PR antibodies (Millanta *et al.,* 2005a);

• nuclear stain in more than 10% of tumour cells for anti-p63 antibody (Ramalho *et al.*, 2006).

Molecular taxonomy

The application of the panel allowed cases to be grouped into five molecular subtypes according to a modified algorithm (Sassi *et al.*, 2010) as follows:

• Luminal-A: ER+ and/or PR+, c-erbB-2–, regardless of CK5/6, CK14, p63 staining.

• Luminal-B: ER+ and/or PR+, c-erbB-2+, regardless of CK5/6, CK14, p63 staining.

• c-erbB-2 overexpressing: ER–, PR–, c-erbB-2+ regardless of CK5/6, CK14, p63 staining.

• Basal-like: ER–, PR–, c-erbB-2–, CK5/6+ and/or CK14+ and/or p63+.

• Normal-like: negative to all markers

Results

Diagnosis

All individual data are listed in Table 2. The first three cases were all from European shorthair feline samples classified as tubulopapillary carcinomas with different metastatic sites. In the first case metastases were present in locoregional lymph node and lung (Fig. 1). The second case developed metastases in locoregional lymph node, lung and muscle. The last feline case presented metastases in locoregional lymph node, lung and spleen. Canine samples were collected from a German Shepherd and a Hungarian Hound. The two canine mammary tumours were histologically diagnosed as anaplastic and tubulopapillary carcinoma respectively with metastases in locoregional, deep, lombo-aortic lymph node, lung, ovary and adrenal gland in the first case and in the lung and brain in the second carcinoma (Fig. 2). At

the time of the investigation no records were available for the involvement of the locoregional

lymph node of the second carcinoma (Table 2).

Table 2: Individual data and molecular phenotypes

N°	1	2	3	4	5
SPECIES	Feline	Feline	Feline	Canine	Canine
BREED	European	European	European	German Shepherd	Hungarian Hound
AGE (years)	NA	NA	19	10	11,8
HISTOTYPE	Tubulopapillary carcinoma	Tubulopapillary carcinoma	Tubulopapillary carcinoma	Anaplastic carcinoma	Tubulopapillary carcinoma
MAMMARY TUMOUR	c-erbB-2 overexpressing	c-erbB-2 overexpressing	c-erbB-2 overexpressing	c-erbB-2 overexpressing	c-erbB-2 overexpressing
LOCOREGIONAL LYMPH NODE METASTASIS	c-erbB-2 overexpressing	Basal-like	c-erbB-2 overexpressing	c-erbB-2 overexpressing	NA
PULMONARY METASTASIS	c-erbB-2 overexpressing	c-erbB-2 overexpressing	NP	c-erbB-2 overexpressing	Basal-like
BRAIN METASTASIS	NP	NP	NP	NP	c-erbB-2 overexpressing
LOMBO-AORTIC LYMPH NODE METASTASIS	NP	NP	NP	c-erbB-2 overexpressing	NP
DEEP INGUINAL LYMPH NODE METASTASIS	NP	NP	NP	c-erbB-2 overexpressing	NP
KIDNEY METASTASIS	NP	NP	NP	NP	NP
SPLEEN METASTASIS	NP	NP	c-erbB-2 overexpressing	NP	NP
MUSCLE METASTASIS	NP	c-erbB-2 overexpressing	NP	NP	NP
OVARY METASTASIS	NP	NP	NP	c-erbB-2 overexpressing	NP
ADRENAL GLAND METASTASIS	NP	NP	NP	c-erbB-2 overexpressing	NP

NA= not available; NP= not present

Immunohistochemistry

None of the cases showed positivity to hormone receptors ER and PR. C-erbB-2

overexpression was observed in the primary sites of all five cases, in locoregional lymph node

metastasis (3 out of 4 cases: 2 cats and 1 dog) and in distant metastases (9 locations: 4 in cats

and 5 in dogs). Considering the two cases (locoregional lymph node metastasis of case no.2

and pulmonary metastasis of case no.5) with hormone receptors and c-erbB-2 negative

staining, the expression of antibody anti-CK5/6, CK14 and p63 was evaluated. The pulmonary metastasis of case no.5 stained only for CK14, with the other three antibodies not being expressed. The locoregional lymph node metastasis of case no. 2 showed positive staining for CK14 and negative staining for CK5/6 and p63.

Molecular taxonomy

Based on the modified algorithm (Sassi *et al*., 2010), only two out of the five different molecular phenotypes were obtained in the primary mammary tumours and their locoregional lymph node and distant metastases in the dogs and cats examined. In the feline cases, all the primary tumours, lymph node metastasis and distant metastases were diagnosed as c-erbB-2 overexpressing phenotype (Fig. 1) with the exception of the locoregional lymph node metastasis in case no. 2 that was diagnosed as basal-like. The two canine cases were classified as c-erbB-2 overexpressing phenotype in the primary site, whereas the lymph node metastasis and distant metastases except the pulmonary metastasis of case no. 5 were diagnosed as basal-like (Table 2) (Fig. 2).

Relationship between molecular phenotype in the primary mammary tumour, its related lymph node and distant metastases

Concordance (the same phenotypes in the primary mammary site and its related lymph node and distant metastases), was found in three out of the five cases (all c-erbB-2 overexpressing phenotypes) (Fig. 1). Two cases showed discordance with the locoregional lymph node (case no. 2, cat, Table 2) and pulmonary distant metastases (case no. 5, dog, Table 2) showing a different phenotypic profile from the primary tumour (c-erbB-2 overexpressing *vs* basal-like) (Fig. 2).

Discussion

In human medicine, molecular phenotypes are determined in both the primary tumour site and in metastases to minimize any therapeutic margin of error if the primary phenotype differs from that of the metastatic lymph node and/or systemic metastases. In breast cancer (Sorlie *et al*., 2003) and lately canine (Beha *et al*., 2012; Gama *et al*., 2008; Sassi *et al*., 2010) and feline (Brunetti *et al*., 2013) mammary neoplasms, the molecular portrait-based classification system has been adopted as a valid tool to evaluate predictive-prognostic models and/or target therapies (Peppercorn *et al*., 2008). Many investigations have demonstrated that different molecular phenotypes resulted in different prognoses and target treatments. Overall- and relapse-free survival times were longer in luminal tumours compared to c-erbB-2overexpressing, basal and normal-like phenotypes (Gama *et al*., 2008; Sorlie *et al*., 2003).

Metastasis to the regional lymph node is considered one of the most important prognostic factors, representing an early step in metastatic spread (Lester, 2010) and the development of distant metastases can be fatal in canine and feline mammary carcinomas (Klopfleisch *et al*., 2010; Morris *et al*., 2008).

The molecular phenotypes of 21 primary feline mammary tumours were determined and compared to their related lymph node metastases obtaining three out of the five known phenotypes (luminal B, c-erbB-2 overexpressing, basal-like) both in the primary neoplasm and in the related lymph node metastasis with 57.1% concordant cases and 42.9% discordant cases (Brunetti *et al*., 2013). The same comparison was studied in 20 bitches and the results disclosed four molecular phenotypes (luminal A, luminal B, c-erbB-2 overexpressing, basal-like) in the primary tumour and all five phenotypes in the lymph node metastasis (luminal A, luminal B, c-erbB-2 overexpressing, basal-like and normal-like) with concordance in 65% of cases and discordance in the remaining 35% (Beha *et al*., 2012).

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The results of the present study in feline and canine carcinomas differed from the above findings since all five primary mammary tumours displayed the same c-erbB-2 overexpressing phenotype. The phenotype was maintained in all the lymph nodes and distant metastases except for one lymph node and one lung metastasis that were discordant with respect to the primary tumour. A possible explanation for the prevalence of the c-erbB-2 overexpressing phenotype in primary tumours is likely to be the small number of cases reported in the present study, but it could also result from the higher malignancy of the c-erbB-2 overexpressing phenotype than the luminal phenotypes, as already demonstrated in human medicine (Perou *et al*., 2000; Sorlie *et al*., 2003). In fact, a previous study demonstrated that ER and/or PR could be lost when the neoplasm metastasizes (Wu *et al*., 2008). The present findings are in accordance with the results reported for cats (Brunetti *et al*., 2013; Millanta *et al*., 2005b) that had a high frequency of c-erbB-2 overexpression particularly in the lymph node metastases, and the well-known malignancy (Misdorp *et al*., 1999) of feline mammary carcinomas. Therefore, it can be assumed that the c-erbB-2 overexpressing phenotype is associated with an increased risk of metastases as in human breast cancer (Aleskandarany *et al*., 2012; Arnedos *et al*., 2012; Fountzilas *et al*., 2012; Steinman *et al*., 2007). Different studies on dog tumour phenotypes (Gama *et al*., 2008; Ressel *et al*., 2013) showed a higher survival rate and lower aggressiveness associated with c-erbB-2 overexpressing phenotypes, which differed substantially from the results obtained in this investigation so that more cases are required before making any assumptions or comparisons. An overall concordance for c-erbB-2 overexpressing phenotypes between primary tumours and metastases was found in female dogs and cats. This result is in agreement with the cited study on feline tumours (Brunetti *et al*., 2013), where a phenotypic concordance of c-erbB-2 overexpressing between primary feline mammary tumour and its related lymph node metastasis was found in 38% of cases with 16 out of 21 primary

mammary tumours presenting the c-erbB-2 overexpressing phenotype in their related lymph node metastases.

Phenotypic concordance of the primary tumour and its related lymph node and distant metastases could be explained by an intrinsic and early metastatic capability of the dominant phenotype in the primary tumours that is maintained in their distant metastases (Weigelt *et al*., 2005).

Planning an anti-c-erbB-2-targeted therapy in veterinary medicine, particularly in female cats bearing mammary carcinomas could therefore have a specific therapeutic value to counteract metastases. Given the affinity of phenotypes to metastasize in different sites, the present results are in agreement with in literature reports on breast cancer showing that the c-erbB-2 overexpressing phenotype has an increased tendency to metastasize to distant (Aleskandarany *et al*., 2012; Fountzilas *et al*., 2012) and locoregional organs (Blows *et al*., 2010).

Two cases in our study presented discordance when the primary site phenotype differed from that or those in the lymph node and/or distant metastases. Case no.2, a cat, presented c-erbB-2 overexpression in both the primary mammary phenotype and distant pulmonary and muscle metastases, but this changed to the basal-like phenotype in the locoregional lymph node metastasis. These results coincide with those obtained in a human breast cancer study where locoregional relapse was associated with the triple negative phenotype (Fountzilas *et al*., 2012). The second discordant case, no.5, was a dog presenting the c-erbB-2 overexpressing phenotype in the primary mammary tumour and the same phenotype in the brain metastasis that became basal-like in the lung metastasis. In human medicine, brain metastasis is more common in triple negative phenotypes (basal and/or normal-like phenotype) (Fountzilas *et al*., 2012; Kennecke *et al*., 2010), and in the c-erbB-2 phenotype (Lester, 2010), in accordance with our case no.5. The basal-like pulmonary metastasis showed overlaps with some reports of breast cancer (Arnedos *et al*., 2012; Kennecke *et al*., 2010; Peng, 2012). However, these

similarities do not identify a standard behavior of the different phenotypes in various metastatic sites since human literature reported indications of cases but not a known behavior. The two discordant cases may be explained by the loss of one or more receptors or by the selection of a subpopulation with an intrinsic program of transition to a different phenotype enhancing their ability for heterotypic interaction and survival proliferation in distant organs (Aleskandarany *et al*., 2012) as Darwinian (Nakshatri *et al*., 2009) evolution.

In canine and feline literature (Brunetti *et al*., 2013; Beha *et al.,* 2012) a greater rate of discordance regarding the relationship between the phenotype of the primary tumour and lymph node metastasis is reported. Otherwise, in this study, among the primary tumour and distant metastases a prevalence of concordant cases is observed.

Conclusions

In conclusion, this study, although limited to five cases, confirmed the existence of both biological phenomena of concordance and discordance in metastatic sites. The prevalence of concordance between primary and metastatic sites supports the predictive therapeutic value of the primary tumour phenotype, minimizing any margin of error which can occur in rare discordant cases.
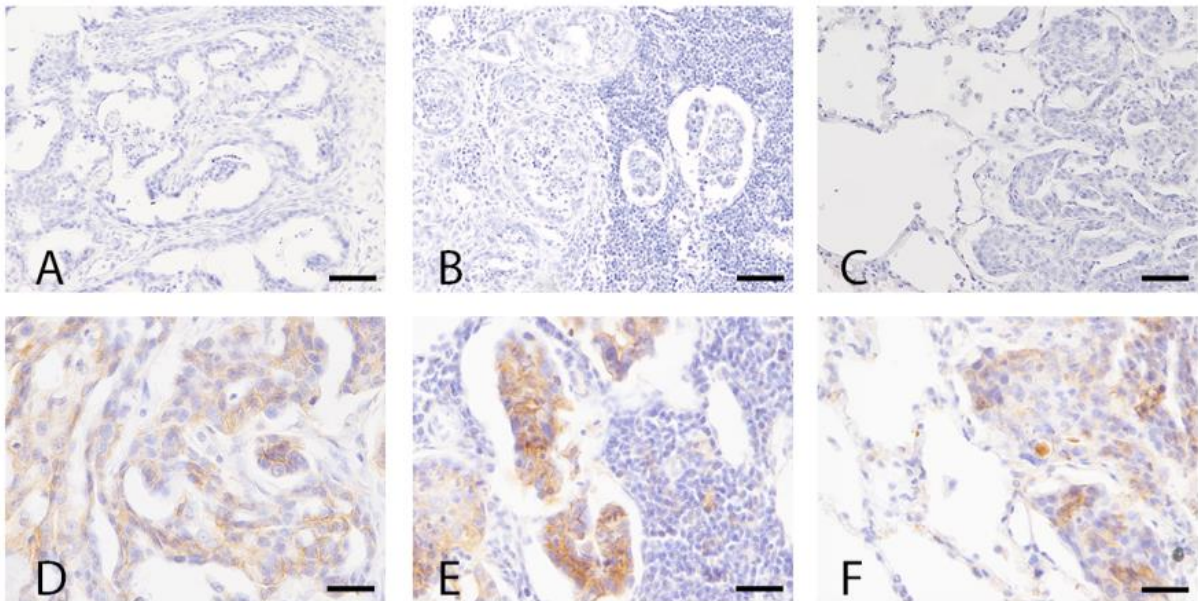
Fig. 1: Cat: Concordance between primary mammary tumour and locoregional and distant metastases. C-erbB-2 overexpressing concordant case with PR– and c-erbB-2+ [A, D (400x. Bar, 38μm)] in the primary neoplasia, PR– and c-erbB-2+ [B, E(400x. Bar, 38μm)] in the lymph node metastasis and PR– and c-erbB-2+ [C, F (400x. Bar, 38μm)] in the pulmonary metastasis. 200x. Bar, 76μm
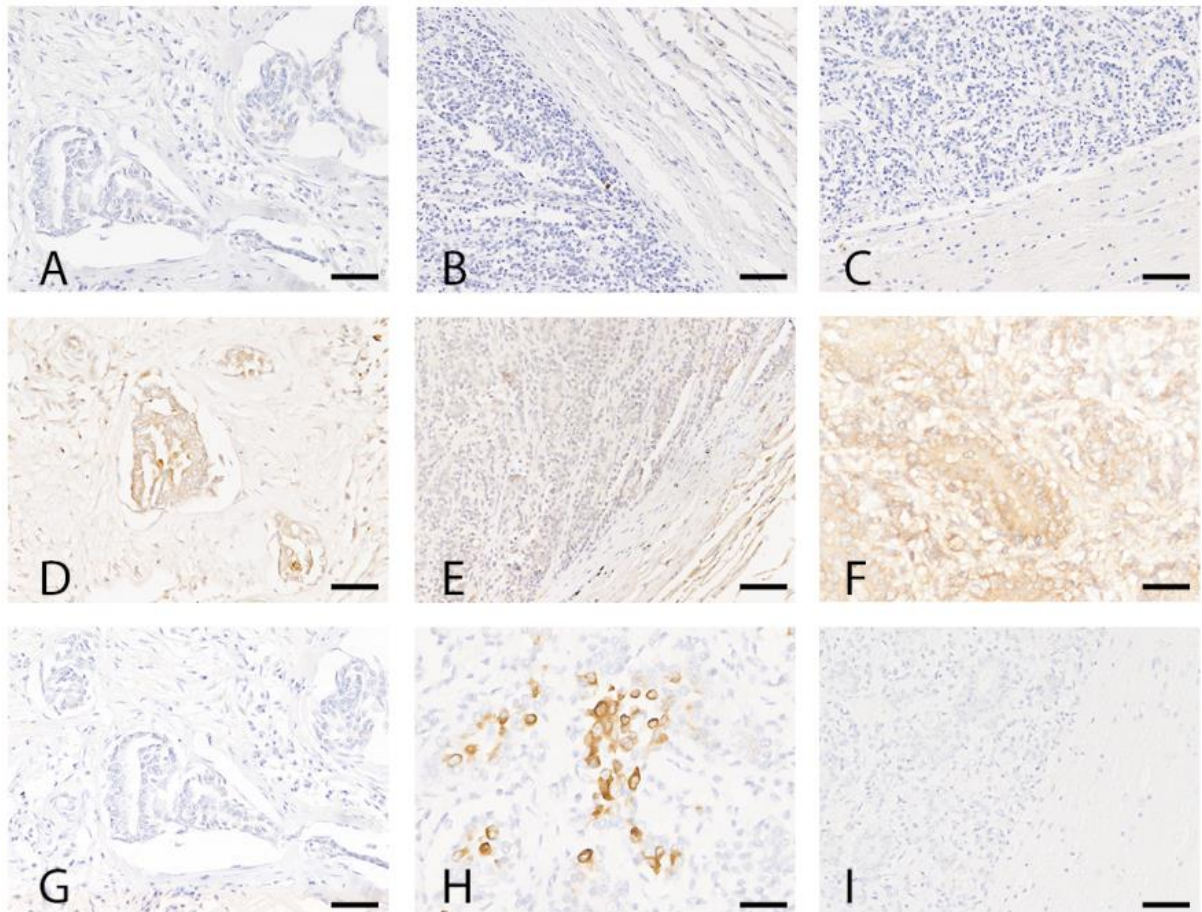
Fig. 2: Dog: Discordance between primary mammary tumour and distant metastases. Discordant case showing c-erbB-2 overexpressing phenotype ER–, c-erbB-2+, CK14– (A, D, G) in the primary tumour becoming basal-like ER–, c-erbB-2–, CK14+ [B, E, H(400x. Bar, 38μm)] in the pulmonary metastasis and c-erbB-2 overexpressing phenotype ER–, c-erbB-2+, CK14– [C, F (400x. Bar, 38μm), I] in the brain metastasis. 200x. Bar, 76μm

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1. Beha G, Muscatello LV, Brunetti B, Asproni P, Millanta F, Poli A, Benazzi C, Sarli G. Molecular Phenotype of Primary Mammary Tumours and Distant Metastases in Female Dogs and Cats. J Comp Pathol. 2013 Sep 20. doi:pii: S0021-9975(13)00132-1. 10.1016/j.jcpa.2013.07.011. [Epub ahead of print].

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FURTHER RESEARCH ON CANINE MAMMARY TUMOURS

4. MORPHOLOGY OF THE MYOEPITHELIAL CELL: IMMUNOHISTOCHEMICAL CHARACTERIZATION FROM RESTING TO MOTILE PHASE

Research Article

Morphology of the Myoepithelial Cell: Immunohistochemical Characterization from Resting to Motile Phase

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Introduction

Mammary gland tumours of dogs are formed by both epithelial (epithelium and myoepithelium) and mesenchymal components. The origin of the mesenchymal cells is still debated. The elevated frequency of tumours showing myoepithelial or basal cell proliferation is a unique feature of canine mammary tumours [1]. In the normal mammary gland, the lumina are delimitated by an inner layer of polarized epithelial cells resting on two outer or basal layers of epithelial and myoepithelial cells [2]. Both basal and myoepithelial cells synthesize the basement membrane of ducts and alveoli and form a structural barrier between the luminal epithelial cells and the surrounding stroma [3]. In ducts, myoepithelial cells form a nearly continuous layer of cells oriented parallel to the long axis of the ducts. This layer surrounds the luminal epithelial cells and separates them from the basement membrane and the stroma. In alveoli, the myoepithelial cells are discontinuous, forming a basket-like

network around the alveoli, allowing some luminal epithelial cells to contact the basement membrane directly [3–5]. Therefore, the myoepithelium is not only located in an ideal position to communicate between these two compartments, but it is also positioned to provide important regulatory signals for the maintenance of normal cell structure [5]. Based on immunohistochemistry, the three layers of cells of the normal mammary gland display different markers: the luminal epithelium is labeled by CK19, and the basal cells and myoepithelial cells are stained by CK5/6 [6] and CK14 [2] and p63, Alpha-SMA, and VIM [2]. Myoepithelial cells are contractile elements exhibiting a combined epithelial and smoothmuscle immunoprofile. Themarkers mentioned above are expressed in the cytoplasm, except for p63 which is a nuclear marker [1]. The myoepithelial cell layer is the sole source of tumour suppressor p63, which is significantly inhibited on proliferation and invasion of associated tumour cells [7]. In addition, basal myoepithelial cells in the normal mammary gland are occasionally labeled by ER antibody [8], which is used for the molecular-based classification of canine mammary tumours [9, 10]. Distinct myoepithelial cell morphologies can be recognized in canine complex and mixed tumours: resting and proliferative suprabasal myoepithelial cells and spindle and stellate motile interstitial myoepithelial cells. Suprabasal cells are located between the basement membrane and the luminal epithelium and exhibit flattened spindle (resting cells) or polygonal morphologies (proliferative cells). Interstitial cells are frequently arranged in solid nests apposed to epithelial elements or isolated in the interstitium [1, 11]. Spindle and stellate myoepithelial cells differentiate toward a more general contractile phenotype [12]. Interstitial myoepithelial cells may eventually become fibroblast-like cells, showing only VIM immunoreactivity [11]. The myoepithelial differentiation may culminate in the formation of various mesenchymal tissues, including cartilage and bone in canine mammary mixed tumour.

The acquisition of typical features of mesenchymal cells is likely to originate through epithelial-mesenchymal transition (EMT). EMT is a biological phenomenon that allows a polarized epithelial cell, which normally interacts with the basement membrane via its basal surface, to undergo multiple biochemical changes enabling it to assume the traits and functions of mesenchymal cells [13].

Aim

This paper will focus on various aspects of myoepithelial cells and mammary tumours in dogs, specifically (1) characterization of the four different myoepithelial cell morphological types in the normal and neoplasticmammary gland using a panel of antibodies and (2) the immunohistochemical changes in myoepithelial cells from an epithelial to a mesenchymal phenotype.

Materials and Methods

Samples

Mammary gland specimens of 29 female dogs were retrieved fromthe database of the Anatomopathological Service of the Faculty of Veterinary Medicine of Bologna. The subjects belonged to different breeds: mongrel (n = 13), German shepherd (n = 3), Poodle (n = 3), Yorkshire Terrier (n = 3), Dachshund (n = 2), Setter (n = 1), Pointer (n = 1), Cocker spaniel (n = 1), Schnauzer (n =1), and Siberian Husky (n = 1); they were all females, with an average age of 9.20 ± 2.28 years (mean ± SD). The tumours consisted of: 3 benign myoepithelial tumours, 3 malignant myoepithelial tumours, 7 carcinomas in benign mixed tumours, and 16 complex carcinomas (the last two groups were differentiated by the presence of cartilage and/or bone in the mixed tumours). In addition, 29 specimens from normal mammary glands

of the same tumour line and 3 mammary samples from 3 healthy nonmammary tumour bearing female dogs were evaluated. Tumours were classified according to Misdorp et al. [14] and Goldschmidt et al. [15] into benign myoepithelial tumours: a rare neoplasm composed of myoepithelial cells arranged in short bundles admixed with an extracellular fibrillar basophilic material; malignant myoepithelial tumours: different from the benign variant with more polymorphic myoepithelial cells; complex carcinoma: a carcinoma composed of both luminal epithelial and myoepithelial components; carcinoma in benign tumour: a tumour with foci of malignant-appearing epithelial cells or distinct nodules of such cells occurring together with mesenchymal cells that have produced cartilage and/or bone possibly in combination with fibrous tissue.

Immunohistochemistry

Four μm thick sections were cutfrom formalin-fixed paraffin-embedded blocks containing representative tumour samples. Immunohistochemistry for the following markers was done on these tissues: CK19, ER, CK5/6, CK14, VIM, Alpha-SMA, p63. Sections were dewaxed in toluene and rehydrated. Endogenous peroxidase was blocked by immersion in 0.3% hydrogen peroxide for 20min. Sections were then rinsed in Tris buffer. Antigen retrieval was performed with citrate buffer (2.1 g citric acid monohydrate/liter distilled water), pH 6.0 (except for CK5/6 and ER, which used EDTA, pH 8.0), and heating for two 5 min periods in a microwave oven at 750 W, followed by cooling at room temperature for 20 min. The primary antibodies are summarized in Table 1. All primary antibodies were incubated overnight at 4∘C, followed by a commercial streptavidin-biotin-peroxidase technique (LSAB Kit, Dako, Amsterdam, The Netherlands). Diaminobenzidine (0.05% for 10min at room temperature) was used as chromogen. Slides were counterstained with Papanicolaou's hematoxylin.

As a negative control, the primary antibody was replaced with an irrelevant, isotype-matched antibody to control for nonspecific binding of the secondary antibody. Positive tissue controls using the same IHC protocols included canine normal mammary gland (anti-CK19, -ER, -CK14, -VIM, Alpha-SMA, -p63 antibodies) and canine skin (anti-CK5/6). The number of positive cells by each marker was calculated semiquantitatively: − = no stained cells, ± = less than 5% positive cells, + = 5–50% positive cells, ++ = more than 50% positive cells. Cases were considered positive for ER when nuclear staining was observed in at least 5% tumour cells [16].

Table 1: Primary antibodies, resources and dilutions used in immunohistochemistry

Antibody (anti-)	Clone	Manufacturer	Dilution
P63	4A4	Dako (Glostrup, Denmark)	1:50
Alpha-SMA	1A4	Dako (Glostrup, Denmark)	1:100
Cytokeratin 19	BA17	Dako (Glostrup, Denmark)	1:50
Cytokeratin 14	Ab-1 (LL002)	NeoMarkers (Fremont, Ca)	1:300
Cytokeratins 5/6	D5/16B4	Zymed (South San Francisco, Ca)	1:100
VIM	V9	Dako (Glostrup, Denmark)	1:100
ER	1D5	Dako (Glostrup, Denmark)	1:25

Results

Four types of myoepithelial cells were recognized on the basis of their morphology. The resting subtype exhibitedvthe elongated features of spindle cells in close contact withvluminal epithelial cells as well as proliferating suprabasal cellsvthat instead showed a polygonal shape (Figure 1(a)). The interstitialmotile cells were observed both forming nests (the spindle type lined nests and the stellate cells constituted the nest core) and isolated in the interstitium (Figure 2(a)).

Normal Mammary Gland

In the 3 control cases, all resting and proliferative suprabasal myoepithelial cells were labeled by p63, CK14, Alpha-SMA, and VIM. Resting and proliferative suprabasal myoepithelial cells did not express CK19 in any of the cases.

Table 2: Immunohistochemical results for suprabasal and motile myoepithelial cells

Type of lesion	Cell morphology		p63	CK14	CK5/6	CK19	Alpha-SMA	VIM	ER
Normal mammary gland (n = 3)°	Suprabasal	resting	++	++	+	–	++	++	+
		proliferative	++	+	+	–	++	++	+
Mammary tissue in the same tumor line (n = 29)°	Suprabasal	resting	++	++	++	–	++	++	+(12/29)
		proliferative	++	+	+	–	++	++	+(12/29)
Benign myoepithelioma (n = 3)§	Motile	spindle	–	±(2/3)	–	–	–	++	–
		stellate	–	–	–	–	–	++	–
Malignant myoepithelioma (n = 3)§	Motile	spindle	–	±	–	–	–	++	–
		stellate	–	–	–	–	–	++	–
Carcinoma in benign-mixed tumor (n = 7)	Suprabasal	resting	++	++	+(5/7)	–	++	++	+(4/7)
		proliferative	++	+/±(2/7)	+(3/7)	–	++	++	+(4/7)
	Motile	spindle	–	±(2/7)	+(3/7)	–	±(5/7)	++	+(3/7)
		stellate	–	–	–	–	–	++	+(3/7)
Complex carcinoma (n = 16)	Suprabasal	resting	++	++ (15/16)	+(11/16)	–	++	++	+(7/16)
		proliferative	++	+(15/16)	+(7/16)	–	++	++	+(7/16)
	Motile	spindle	–	±(2/16)	+(7/16)	–	±(5/16)	++	+(6/16)
		stellate	–	–	–	–	±(2/16)	++	+(6/16)

* –: no stained cells; ±: less than 5% positive cells; +: 5–50% positive cells; ++: more than 50% positive cells.
°: the motile phonotype is not updated because not present.
§: the suprabasal phonotype is not updated because not detectable around luminal cells.

Mammary Tissue from the Same Line of the Mammary Tumour.

In the 29 normal tissues in the same line as the tumours, all resting and proliferative suprabasal myoepithelial cells were labeled by p63, CK14, Alpha-SMA, and VIM. CK5/6 was positive in all but four cases and ER was detected in 12 cases. CK19 expression was only observed in the luminal epithelium. Myoepithelial motile interstitial cells were not observed. All the results are summarized in Table 2.

Mammary Tumours

Immunohistochemical results for suprabasal (Figure 1) and motile cells (Figure 2) using p63, CK14, CK5/6, CK19, Alpha-SMA, VIM, and ER are summarized in Table 2. CK5/6 labeled: suprabasal resting cells in 16 of the 29 cases (5 carcinomas in benign mixed tumours and 11 complex carcinomas). CK 5/6 also labeled proliferativesuprabasal and spindle motile cells in 10 cases (3 carcinomas in benign mixed tumours and 7 complex carcinomas). Stellate motile cells were present in 25 cases (1 benign myoepithelial tumour, 3 malignant myoepithelial tumours, 5 carcinomas in benign mixed tumours and 15 complex carcinomas) but were negative for CK5/6. Cartilage in mammary mixed tumours was always negative. CK14 showed positivity in 23 cases in resting cells (with the exception of 1 complex carcinoma and benign and malignant myoepithelial tumours), in 22 cases in proliferative cells (6 carcinomas in benign mixed tumours and 15 complex carcinomas) with a trend to lose the expression when these cells had acquired the more motile phenotype of spindle cells (positive in 2 benign and 3 malignant myoepithelial tumours, 2 carcinomas in benign mixed tumours and 2 complex carcinomas). Chondrocytes of mixed tumours were negative. VIM was positive in suprabasal cells in the 23 cases of carcinoma in benign tumour and complex carcinoma and in motile myoepithelial cells in all 29 cases. Stromal cells were positive in all cases. Cartilage was VIM positive in all 7 carcinomas in benign-mixed tumours. Alpha-SMA labeled resting and proliferative suprabasal myoepithelial cells in 23 carcinomas in benign-mixed tumours and complex carcinomas. The spindle cells in 10 cases (5 carcinomas in benign mixed tumour and 5 complex carcinomas) showed positivity for Alpha-SMA. In each case, except for two complex carcinomas, stellate cells were negative. Stroma showed positivity in only 6 cases (1 carcinoma in benignmixed tumour and 5 complex carcinomas). P63 was detected in resting and proliferative suprabasal myoepithelial cells of the 23 cases of carcinoma in benign

mixed tumour and complex carcinoma. All spindle and stellate motile cells were negative. Stromal cells and cartilage were negative. ER expression was present in 11 suprabasalmyoepithelial cells (4 carcinomas in benign-mixed tumours and 7 complex carcinomas); spindle and stellate motile cells were positive in 9 cases (3 carcinomas in benign-mixed tumours and 6 complex carcinomas). Cartilage of mixed tumours was negative. Resting and proliferative suprabasal and spindle/stellate motile myoepithelial cells did not express CK19 in any of the tumours examined. Cartilage of mixed tumours was also negative for CK19.

Discussion

Based on the findings of Gama *et al*. [1] and Tateyama *et al*. [11], four morphological types of myoepithelial cells are present in the mammary gland: resting and proliferative suprabasal myoepithelial cells lining alveoli and ducts and spindle and stellate interstitial motile cells, which lie in the interstitial space where they may be arranged in nests. Myoepithelial markers, such as p63, CK5/6, CK14, Alpha- SMA, and VIM, proved to be valuable diagnostic adjuncts to facilitate the evaluation of complex and mixed proliferations. CK19 is considered the gold standard marker for luminal epithelium and was used to avoid any misdiagnosis with myoepithelial cells. Because of cross-reactivity patterns and the fact that lesional foci are typically minute, none of the myoepithelial markers enjoyed 100% sensitivity and specificity for myoepithelial cells. As such, at least 2 markers should be used to evaluate any given focus [17]. Based on our results, the best marker for suprabasal cells was p63 especially in association with CK14, which was limited to mature (basal) myoepithelial cells and, to a lesser extent followed by CK5/6, Alpha-SMA and VIM (Figure 1). However, CK5/6 also marked luminal epithelial cellsmaking it difficult to distinguish them fromproliferative suprabasal myoepithelial cells [2]. Morphologically, both epithelial and myoepithelial cells

may have a polygonal shape. A characteristic of both CK14 and CK5/6, but not of p63, alpha-SMA, and VIM, was their reduced expression in myoepithelial cells in the suprabasal proliferative state. CK14, CK5/6, and p63 expression was gradually lost in cells in the spindle and stellate motile state. Alpha-SMA and VIM were present in spindle motile myoepithelial cells with different degrees of intensity. Only VIM proved to be a consistent marker for stellate motile myoepithelial cells. In this study, the stellate motile myoepithelium was arranged in nests and lined by resting cells presumably of alveolar origin. This feature may support the idea that the nests of stellate motilemyoepithelial cells, which have lost expression of the main myoepithelial suprabasal markers, but retained affinity for VIM, are the precursors of cartilage, indicating that these cells have completed their transformation into mesenchymal elements. In benign and malignant myoepithelial tumours, VIM labeling in all cases, loss of all other suprabasal myoepithelial markers, and the scant positivity to CK14 in spindle cells were indicative of a prevailing expression of the myoepithelium motile state and a possible passage from simple myoepithelial cells to mesenchymal fibroblasts. In our study, evidence of the myoepithelial cells shifting to a mesenchymal phenotype, shown by the loss of CK14, CK5/6, and p63 expression, was reinforced by the discontinuous labeling of spindle cells for Alpha-SMA, a marker of both myoepithelial cells and myofibroblasts, which was completely lost in stellate motile cells that have supposedly become fibroblasts. Further confirmation studies by Tsuda *et al*. [18] reported the occurrence of myofibroblasts with remnants of CK14 expression (described as "converted myoepithelial cells"). In the cases examined in the present study, CK14 progressively faded, therefore indicating a loss of the (myo-)epithelial phenotype. These results support the EMT hypothesis involving a myoepithelial-like state [19], which undergoes a myoepithelial mesenchymal transition (MMT). This hypothesis was confirmed in the dog by Gartner *et al*. [20] who stated that in

mammary tumours one of the steps in the evolution ofmesenchymal cells involves the expression of typical myoepithelial traits. An interesting result of the present study was the positivity to ER found in 12/29 suprabasalmyoepithelial cells and 9/29 stellate cells of carcinoma in benign-mixed tumours and complex carcinomas. Two isoforms of ER receptors have been described, namely, ER-α and ER-β, the latter being the only form expressed in the nuclei of isolated basalmyoepithelial cells [8]. The antibody used in the present investigation was inclusive of both isoforms: both luminal and basal/stellate cells were labeled, presumably luminal cells by ER- β and basal/stellate cells by ER-α.

Conclusions

In conclusion, the suprabasal myoepithelial cells were well characterized by p63 and CK14 and to a lesser extent by the other marker used. The motile myoepithelial cells are instead characterized by Alpha-SMA and VIM and loss of CK14, CK5/6, and p63 (Figure 2). The present study also demonstrated ER in both luminal epithelial and suprabasal/stellate myoepithelial cells (the latter in about half of the cases) and that ER expression is not influenced by the resting/motile phase. Therefore, in serial or multistained sections, immunohistochemistry to ER in combination with p63 and CK14 may serve to avoid erroneous identification of luminal or myoepithelial cells in canine mammary tumours.

The trend of preserved Alpha-SMA and VIM expression in spindle cells, and only VIM positivity in stellate motile cells as well as the decreased p63 expression in both motile types, supports the hypothesis of the EMT involving a myoepithelial-like state [19] in MMT. The spindle motile cell could be considered an earlier transformation than the stellate cell towards a mesenchymal phenotype.
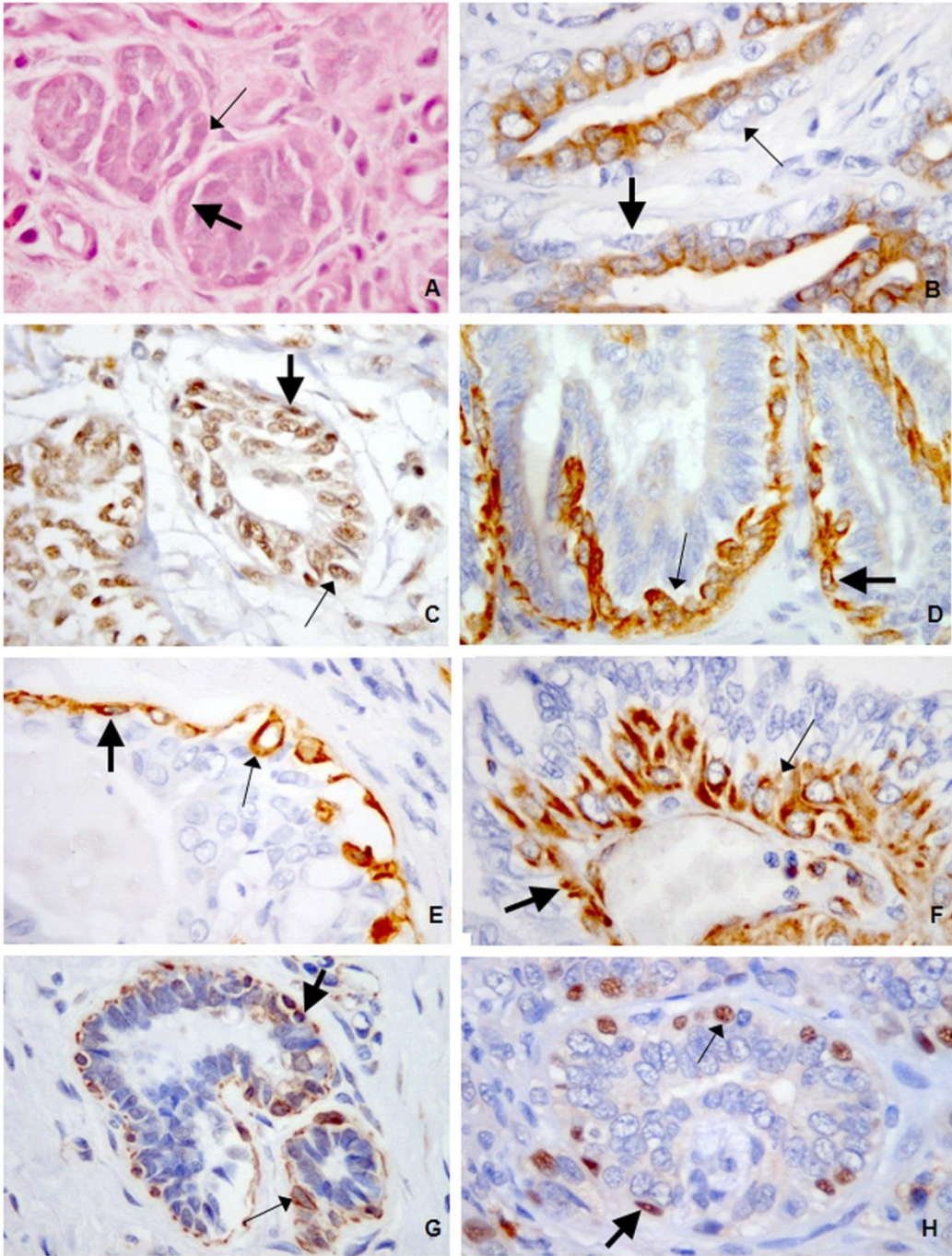
Figures

Fig. 1: Suprabasal myoepithelial cells: resting (thick arrows) and proliferative (thin arrows) cells. Immunohistochemical expression of a panel of antibodies applied by IHC, 63x

(A) Hematoxylin-eosin; (B) anti-CK19 antibodies labeling the cytoplasm; (C) anti-ER antibodies labeling the nuclei; (D) anti-CK 5/6 antibodies labeling the cytoplasm; (E) anti-CK14 antibodies labeling the cytoplasm; (F) anti-VIM antibodies labeling the cytoplasm; (G)

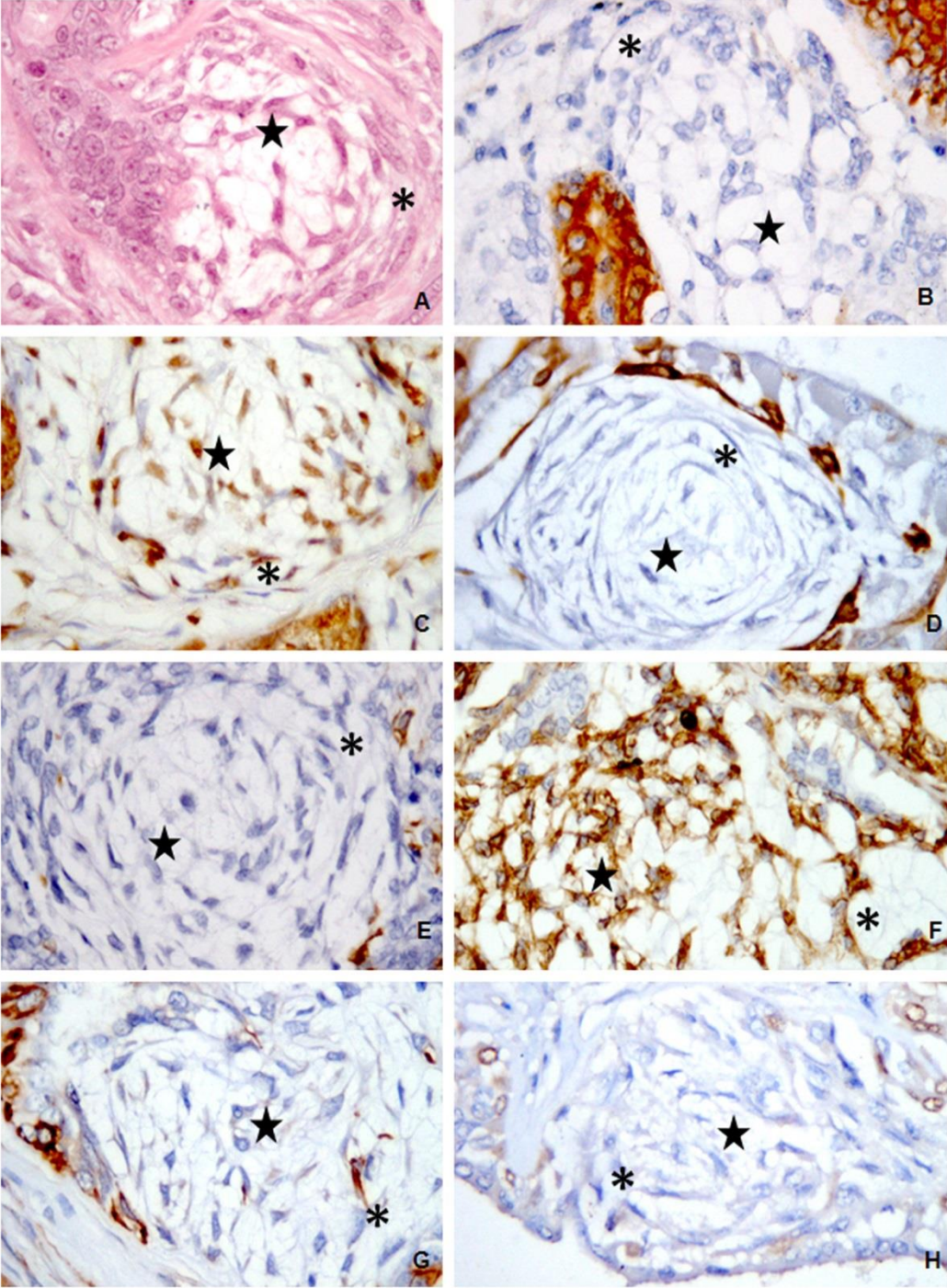anti-Alpha-SMA antibodies labeling the cytoplasmic membrane; (H) anti-p63 antibodies labeling the nuclei.

Fig. 2: Motile myoepithelial cells: spindle (asterisks) and stellate (stars) cells. Immunohistochemical expression of a panel of antibodies applied by IHC. 63x
(A) Hematoxylin-eosin; (B) anti-CK19 antibodies labeling the cytoplasm; (C) anti-ER antibodies labeling the nuclei; (D) anti-CK 5/6 antibodies labeling the cytoplasm; (E) anti-

CK14 antibodies labeling the cytoplasm; (F) anti-VIM antibodies labeling the cytoplasm; (G) anti-Alpha-SMA antibodies labeling the cytoplasmic membrane; (H) anti-p63 antibodies labeling the nuclei.

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5. CD117 EXPRESSION INFLUENCES PROLIFERATION BUT NOT SURVIVAL IN CANINE MAMMARY TUMOURS

CD117 expression influences proliferation but not survival in canine mammary tumours

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Introduction

C-Kit is a gene that encodes a membrane-associated tyrosine kinase growth factor receptor (CD117) (Yarden *et al.*, 1987), composed of an extracellular ligand-binding region (5 immunoglobulin-like domains), a single transmembrane spanning region (hydrophobic domain), and a cytoplasmic region which includes both the kinase (ATP-binding and phosphotransferase) and the juxtamembrane domains (Linnekin, 1999; Boissan *et al.*, 2000). CD117 is expressed in various cell types during embryonic development and it promotes the migration, differentiation, proliferation, growth, adhesion, chemotaxis and survival of cells (Chian *et al.,* 2001; Ronnstrand, 2004). CD117 has been shown to be expressed by neoplastic cells as well; in the dog most notably, in gastrointestinal stromal tumors (GIST) (Bettini *et al.,* 2003; Frost *et al.*, 2003; Smith *et al*, 2009), in mast cell tumours (Gil da Costa *et al.,* 2007; Thompson *et al.,* 2011), neoplastic testes (Bush *et al.,* 2011; Thorvaldsen *et al.,* 2012), and

melanocytic tumours (Murakami *et al.,* 2011; Gomes *et al.,* 2012). Few studies have focused on the expression of CD117 in canine mammary tissue, and the difference in its expression between normal tissue and neoplastic benign or malignant change (Kubo *et al.,* 1998; Morini *et al.,* 2004; Sailasuta *et al.,* 2008). Kubo *et al.* (1998) investigated the expression of the canine *c-Kit* oncogene in mammary tumours with the aid of RT-PCR; the results showed a significant higher level of transcription in adenocarcinoma than in malignant mixed tumours. Morini *et al.* (2004) and Sailasuta *et al.* (2008) evaluated CD117 expression using immunohistochemistry. Morini *et al.* (2004) found a weak to moderate cytoplasmic staining in ductal and acinar epithelial cells of the normal canine mammary gland, and a weak to moderate cytoplasmic staining in benign and malignant mammary tumours. Sailasuta *et al.* (2008) found either membranous, cytoplasmic staining or both, but they did not see a statistically significant difference between the CD117 expression and histological type, histological malignancy, nuclear differentiation or grade of mammary gland tumour.

Since *c-Kit* is a proto-oncogene that encodes a transmembrane tyrosine kinase growth factor receptor and stimulates cell proliferation, it plays a crucial role in determining existence of a correlation between *c-Kit* expression and Ki67 index. A correlation between aberrant CD117 expression and increased cell proliferation in canine mast cell tumours has been reported (Gil da Costa *et al.,* 2007; Webster *et al.,* 2007; Thompson *et al.,* 2011). Therefore, the aims of the present study were (1) to characterize the immunohistochemical staining of CD117 in normal and neoplastic mammary tissue of the dog, and (2) to correlate CD117 immunohistochemical results with mammary histotype, histological stage (invasiveness), Ki67 index and patient survival time.

Materials And Methods

Study material

In this study 49 samples of normal and neoplastic canine mammary tissue were examined. The specimens were retrieved from the archive of the Veterinary Pathology Division of the Department of Veterinary Medical Sciences of the University of Bologna. The samples included 8 normal, hyperplastic and dysplastic mammary tissue specimens, 11 cases of benign mammary tumours and 30 cases of malignant mammary neoplasms. Tissue specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections of 4 µm were stained with Haematoxylin–Eosin (H-E) and observed microscopically. The samples were then histologically diagnosed according to the criteria set by Misdorp *et al.,* (1999). In malignant tumours, the histologic stage of invasion was determined according to Gilbertson *et al.* (1983) as follows: stage 0 = tumours without stromal invasion; stage I = tumours with stromal invasion, stage II = tumours with neoplastic emboli in vessels or lymph node metastases (or both). As a follow-up the owners/referring veterinarians of all cases of malignant tumours were contacted in order to obtain information regarding survival time and the cause of death.

Immunohistochemistry

Two consecutive sections were dewaxed in toluene and rehydrated. Endogenous peroxidase was blocked by immersion in 3% hydrogen peroxide for 30 minutes. Sections were then rinsed in Tris buffer, immersed in citrate buffer (2.1g citric acid monohydrate/litre of distilled water), pH 6.0, incubated for 2 cycles of 5 minutes (anti-CD117 antibody) and for 4 cycles of 5 minutes each (anti-Ki67 antibody) in a microwave oven at 750W, and allowed to cool down at room temperature for approximately 20 minutes.

The antibodies (CD117, polyclonal, Dako Cytomation, 1:100 dilution with phosphate-buffered saline solution and 1% BSA) and anti-Ki67 antibody (clone MIB-1, Dako Cytomation, 1:30 dilution with phosphate-buffered solution) were applied, and the sections were incubated overnight at 4ºC. Sites of primary antibody binding were identified using a

commercial Streptavidin-biotin-peroxidase kit (LSAB KIT, Dako) and diaminobenzidine as the chromogen (0.04% for 10 minutes). The sections were then counterstained with Papanicolaou Haematoxylin, rinsed in tap water, dehydrated and cover-slipped.

Sections of a well differentiated canine mast cell tumour were used as positive controls for CD117 and the basal layer of the epidermis served as an internal positive control for Ki67. Negative controls were prepared by incubating the slides with an isotype-matched nonspecific antibody.

CD117 evaluation

The slides were blindly evaluated by 2 board-certified anatomical pathologists (C.B. and B.B.) for CD117 immunoreactivity. The results were considered positive when a brown labelling of the cells was present, and negative when no immunoreactivity was noted on the section (CD117 expression).

The pattern of positivity of the cells for CD117 was classified as membranous (M) (brown labelling of the cytoplasmic membrane), cytoplasmic (C) (diffuse brown labelling of the cytoplasm) or membranous-cytoplasmic (MC) (simultaneous cytoplasmic and membranous expressions). CD117 extension was the percentage of positively labelled cells (extension) evaluated as follows: 0= negative (0%), 1= focal (1-19%), 2= intermediate (20-49%), 3= diffuse (> 50%) as described by Gomes et al. (2012) modified.

Ki67 index

Ki67 immunoistochemical labelling was blindly evaluated by a single pathologist (G.B.) using a 40x objective to select 5 fields with the highest Ki67 positivity; areas with necrosis or inflammation were avoided. Ki67 index was calculated as the percentage of labelled nuclei

compared with the total nuclear area of the field according to a previously described method (Sarli *et al.*, 2002).

Statistics

The correlation between CD117 expression, CD117 pattern or CD117 extension vs type of mammary lesion, histological classification, histological stage and Ki67 index was tested with the Pearson's chi-square (χ^2) test. Ki67 index was tested with the Shapiro Wilk's W analysis for normality and appeared non-normally distributed. Due to this abnormal distribution, Ki67 index in CD117 expression groups (positive vs negative) and CD117 patterns were tested with the non-parametric Kruskal Wallis ANOVA Median test. Survival analysis was used to compare Kaplan Meyer estimated curves in 2 groups (CD117 expression in the 3 groups (CD117 pattern) or in the 4 groups (CD117 exension) selecting only those cases in which death was due with no doubt to the tumour. In all tests a value of $P<0.05$ was considered to be statistically significant.

Results

The 49 cases were as follows: 3 cases of normal mammary tissue, 5 cases of hyperplasia, 11 benign tumours (4 tubulo-papillary adenomas, 1 simple adenoma, 1 complex adenoma, 3 fibroadenomas, and 2 benign mixed tumours); 30 malignant tumours (10 solid carcinomas, 4 carcinoma in benign tumours, 8 simple tubulo-papillary carcinomas, 5 complex carcinomas, 2 anaplastic carcinomas, and 1 squamous carcinoma). Of these 30 malignant tumours, 2 were found to be histological stage 0, 12 stage I, and 16 stage II. The mast cell tumour used as positive control showed a strong membranous positivity in all neoplastic cells for CD117 expression. Ki67 expression was evident in the nuclei of the basal layer of epidermis.

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Information about the patients' survival time of 22 out of the 30 malignant tumours studied
was retrieved, and in 13 cases death was correlated with the presence of metastasis (Table 1).

Table 1: Individual data

N°	HISTOTYPE	STAGE	CD117 EXPRESSION	CD117 PATTERN	CD117 EXTENSION	Ki67 index	SURVIVAL RATE (MONTHS)
1	Normal		P	M	3	7,9	
2	Normal		P	M/C	3	5,78	
3	Normal		P	C	1	4,27	
4	ADH		P	C	1	3,34	
5	ADH		P	C	1	3,56	
6	ADH		N		0	2,84	
7	Hyperplasia		N		0	6,5	
8	Hyperplasia		P	M	3	6,16	
9	Tubpap adenoma		P	C	1	4,15	
10	Tubpap adenoma		P	C	2	3,07	
11	Tubpap adenoma		P	C	3	2,79	
12	Tubpap adenoma		N		0	3,43	
13	Complex adenoma		N		0	2,7	
14	Simple adenoma		N		0	14,34	
15	Fibroadenoma		P	C	1	5,36	
16	Fibroadenoma		P	M	3	25,07	
17	Fibroadenoma		N		0	1,66	
18	BMT		N		0	3,07	
19	BMT		N		0	8,45	
20	Solid carcinoma	2	P	C	1	9,83	36
21	Solid carcinoma	1	P	C	2	3,85	24
22	Solid carcinoma	2	P	C	2	5,77	NA

23	Solid carcinoma	0	P	M/C	3	8,53	NA
24	Solid carcinoma	1	P	M/C	3	6,49	NA
25	Solid carcinoma	2	P	M/C	3	23,18	3
26	Solid carcinoma	2	P	M/C	3	8,18	24
27	Solid carcinoma	1	P	M/C	3	9,39	24
28	Solid carcinoma	1	N		0	4,26	NA
29	Solid carcinoma	2	N		0	1,86	1
30	CBT	2	P	M/C	3	8,14	24
31	CBT	2	P	M/C	3	13,85	48
32	CBT	1	P	C	3	5,95	1
33	CBT	1	N		0	2,65	48
34	Tubpap carc	2	P	C	1	8,21	12
35	Tubpap carc	1	P	C	2	2,78	6
36	Tubpap carc	1	P	M/C	2	5,58	NA
37	Tubpap carc	2	P	C	3	6,67	1
38	Tubpap carc	2	P	M/C	3	9,22	NA
39	Tubpap carc	2	N		0	1,96	2
40	Tubpap carc	1	N		0	2,45	12
41	Tubpap carc	1	N		0	6,26	NA
42	Complex carc	2	P	C	2	3,67	24
43	Complex carc	0	N		0	2,56	5
44	Complex carc	2	N		0	2,95	6
45	Complex carc	1	N		0	2,13	A
46	Complex carc	1	N		0	1,97	N/A
47	Anaplastic carc	2	P	C	3	8,25	2
48	Anaplastic carc	2	P	C	2	5,51	2
49	Squamous carc	2	P	C	3	8,16	A

ADH: atypical ductal hyperplasia. Tubpap adenoma: tubulo-papillary adenoma. BMT: benign in mixed tumour. CBT: carcinoma in benign mixed tumours. Tubpap carc: tubulo-papillary carcinoma. Comlex carc: complex carcinoma. Anaplastic carc: anaplastic carcinoma. Squamous carc: squamous carcinoma. P: positive. N: negative. M: membranous. C: cytoplasmic. M/C: membranous and cytoplasmic. NA: available. A: alive

Normal mammary tissue and hyperplasia

CD117 expression was observed in the alveolar and ductal mammary epithelium in 6 of the 8 examined normal and hyperplastic mammary tissues. No expression was detected in the myoepithelial and in the stromal cells in any of the cases. The remaining 2 cases (mammary hyperplasia) were negative. The CD117 pattern was M in two cases (1 normal mammary tissue and 1 hyperplasia), C in 3 cases (1 normal mammary tissue and 2 hyperplasias), M/C in 1 normal mammary tissue (Fig. 1a). CD117 extension was focal in 3 cases (1 normal mammary tissue and 2 hyperplasias) and diffuse in the remaining (1 normal mammary tissue, 2 hyperplasias) (Table 1, Fig. 2a, 2d, 2g).

Benign mammary tumours

In benign mammary tumours CD117 was expressed exclusively in the epithelial cells in 5 out of 11 cases. Regarding CD117 pattern, 3 of the 4 tubulo-papillary adenomas were positive with C labelling and 1 was negative. Two positive cases out of 3 of the fibroadenoma type showed a C and a M pattern respectively (Fig. 1b). The remaining fibroadenoma as well the simple adenoma, the complex adenoma, and the 2 benign mixed tumours did not express CD117 . In the positive cases the labelling extension was focal in 2 cases (1 tubulo-papillary adenoma and 1 fibroadenoma), intermediate in a tubulo-papillary adenoma and diffuse in 2 cases (1 tubulo-papillary adenoma and 1 fibroadenoma). All the data are reported in Table 1 and Fig. 2a, 2d, 2g.

Malignant mammary tumours

CD117 immunoexpression was present in 20 of the 30 malignant tumours, namely 8 solid carcinomas, 3 carcinoma in benign mixed tumours, 5 tubulo-papillary carcinomas, 1 complex

carcinoma, 2 anaplastic carcinomas and 1 squamous cell carcinoma. For the labelling location, the positive cells were all epithelial cells except for one case that presented both epithelial and myoepithelial staining (carcinoma in benign tumour). The 8 solid carcinomas displayed different CD117 pattern: C reactivity in 3 cases and M/C in 5 cases (Fig. 1d, 1e, 1f). The 3 carcinomas in benign mixed tumours showed C positivity in one case, and M/C in the other 2 cases (Fig. 1c). The cartilaginous component of the latter 3 cases was negative for CD117 (Fig. 1c). The 5 tubulo-papillary carcinomas displayed C in 3 cases and M/C pattern in 2 cases. The 2 anaplastic carcinoma, complex carcinoma and squamous cell carcinoma showed C expression. The labelling extension was focal in 3 cases (1 solid carcinoma, 1 tubulo-papillary, 1 complex carcinoma), intermediate in 5 cases (2 solid carcinomas, 2 tubulo-papillary carcinomas, 1 anaplastic carcinoma) and diffuse in 12 cases (5 solid carcinomas, 3 carcinomas in benign mixed tumours, 2 tubulo-papillary carcinomas, 1 anaplastic carcinoma, 1 squamous cell carcinoma) (Table 1, Fig. 2).

Correlation between the three CD117 variables and type of mammary lesion, malignant histotypes and histological stage

None of the 3 CD117 variables (CD117 expression, CD117 pattern and CD117 extension) appeared to be associated with the different mammary lesion types (normal/hyperplasia, benign tumours, malignant tumours), histotypes and invasiveness. Only in malignant tumours was an association between CD117 pattern and histological type evident (R=0.37, P=0.043, Pearson test) (Table 2).

Correlation between CD117 expression, pattern, extension and Ki67

All the 3 CD117 variables were significantly associated with Ki67 index (Table 2) (Fig. 3a, 3c, 3e). Comparing the Ki67 value of the CD117 positive cases (CD117 expression) vs those

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negative, only in malignant tumours was a significantly higher Ki67 value apparent (P<0.001, Kruskall-Wallis test) (Fig. 3a). The tumours with both cytoplasmic and membranous staining patterns showed a significantly higher range and higher median value of the Ki67 index compared to cases with only cytoplasmic expression (P<0.001, Kruskall-Wallis test), and were both significantly higher than negative cases (P<0.01, Kruskall-Wallis test) (Fig. 3c). Ki67 showed values increased progressively from negative cases to those presenting diffuse extension staining of CD117 (P<0.001, R=0.61, Spearman test) (Fig. 3e) (Table 2).

Correlation between CD117 expression, pattern, extension and patient survival time

Survival time was available for 22 female dogs bearing malignant tumours. In only 13 cases death was due to the tumour and only in these cases survival analysis was performed. Survival analysis did not reveal any differences in the comparison of the 2 groups of CD117 expression (positive vs negative) (P=0.91 Survival analysis) (Fig. 3b) or the 3 groups of CD117 pattern (membranous, cytoplasmic, cytoplasmic+membranous) (P=0.46 Survival analysis) (Fig. 3d) and the four groups of CD117 extension (absent, focal, intermediate, diffuse) (P=0.85 Survival analysis) (Fig. 3f) (Table 2).

Table 2: R and P values of the Pearson chi square test

	Nmg/Hyp vs BT vs MT		Histotypes of MT		Invasiveness		Ki67 index	
	R	P	R	P	R	P	R	P
CD117 expression	-0.004	0.97	0.21	0.25	-0.30	0.10	-0.35	0.014
CD117 pattern	0.10	0.47	0.37	0.043	-0.16	0.37	-0.55	<0.001
CD117 extension	0.14	0.33	-0.15	0.40	0.25	0.17	0.51	<0.001

Nmg = normal mammary gland; Hyp = hyperplasia; BT = benign tumour; MT = malignant tumour.

Discussion

Normal human breast tissues strongly express the *c-Kit* proto-oncogene product on the cell membrane and/or cytoplasm of alveolar and ductal cells (Chui *et al.,* 1996; Tomasino *et al.,* 2009). Regarding benign and malignant human breast diseases discordant results are reported in literature; in fact, in several studies the *c-Kit* proto-oncogene product was detected heterogeneously with a reduced immunoreactive score in benign lesions and even less represented in breast cancer (Chui *et al.,* 1996; Tomasino *et al.,* 2009; Kondi-Pafiti *et al.,* 2010). Other Authors found an increased expression of CD117 in poorly differentiated breast cancer (Diallo *et al.,* 2006; Kanapathy Pillai *et al.,* 2012).

By now, only few studies have investigated the expression of CD117 in canine mammary tumours (Kubo *et al.,* 1998; Morini *et al.,* 2004; Sailasuta *et al.,* 2008), but with no clear results about the importance of this receptor.

The present study evaluated CD117 expression, pattern and extension in canine mammary tissue (normal and neoplastic), correlating the results not only to different mammary lesions, histotypes, histological stage and survival, but also with a marker of proliferation Ki67.

Considering CD117 expression, the present results, also in agreement with Sailasuta *et al.,* (2008), showed no statistically significant differences between the expression of CD117 (positivity/negativity) and different histotypes. Our results demonstrated a distinctive decrease in CD117 expression in benign tumours comparing with normal tissue (similarly to what seen in human studies), but with no such a distinctive decrease in CD117 expression in malignant tumours as reported in some human breast cancer studies (Chui *et al.,* 1996; Tomasino *et al.,* 2009; Kondi-Pafiti *et al.*, 2010). This may be linked to canine tumour heterogenicity or perhaps because malignant mammary tumours are usually very well differentiated and present a less malignant behaviour than their of the human counterparts.

Regarding the CD117 pattern, in normal mammary glands all three patterns were found. This result may be indicative of functional activity of CD117 irrespective of the type of positivity. In benign tumours, the present results highlighted the absence of simultaneous M/C expression, as the cases were completely C or M. With the progression of the lesions (malignant tumours), C and M/C, but not M only, were present. The decreasing trend of M only expression to the simultaneous M/C expression or even only C can be interpreted as an accumulation of receptor proteins that did not undergo final maturation, or an excessive internalization of activated *c-Kit* and/or an abnormal synthesis of the receptor, preventing it from reaching the transmembrane region as suggested by different authors (Torres-Cabala *et al.,* 2009; Gomes *et al.,* 2012). A significant correlation between CD117 pattern and histotypes of malignant tumours was found (R=0.37, P=0.043, Pearson test) indicating that the localization of this type of receptor may be associated with the morphological type of the malignant mammary carcinoma with an increasing percentage of "only cytoplasmic" pattern from the well differentiated to the less differentiated histotypes.

Analyzing the CD117 extension (percent of positive cells) it can be observed that all the extension types (i.e. decrease of positivity) are present in the different type of mammary lesion except for the "intermediate" in the group of normal mammary tissue and hyperplasia, which could be due to the low number of cases; in addition, the extension of the positivity was not related to the type of mammary lesion and malignant histotypes.

None of the three parameters (CD117 expression, pattern and extension) were shown to be correlated with invasiveness, although, this association has been observed in pancreatic cancer cells (Yasuda *et al.,* 2006).

Similar negative findings occurred correlating the three parameters (CD117 expression, pattern and extension) with survival time, i.e. there was no correlation of CD117 and patient survival.

The usefulness of CD117 results changes when these are correlated with the Ki67 index. Proliferative activity is strictly associated not only to positive/negative CD117 expression, but also to the type of expression (CD117 pattern) and to the percentage of labelling (CD117 extension), suggesting in canine mammary tumours the existence of a link between the presence of the receptor and the proliferative activity. This also suggests that the M, M/C as well as only C expression are able to induce proliferation, with decreasing grades of signal from M to M/C to C only, demonstrated by our results in mammary malignant neoplasms. Gil da Costa *et al.* (2007) as well, in their paper about canine mast cell tumours results have highlighted a strong correlation between C (altered) CD117 immunoexpression and increased cell proliferation. These results suggest that even a C expression pattern is able to partially increase cellular proliferation as assessed by the Ki67 labelling index. As noted above, the different patterns of expression, which have been identified in the juxtamembrane domain of *c-Kit* in certain canine mast cell tumours which are attributed to point mutations, deletions, and duplications (Preziosi *et al.*, 2004), seem to apply to mammary neoplasms as well. It is well-known (Regan *et al.,* 2012) that the *c-Kit* signaling pathway is required for the normal function of mammary epithelial progenitors.

Conclusions

The M, M/C and C expression, existing in the normal mammary gland as well as in neoplastic growth, suggest that in transformed mammary cells the *c-Kit* signalling network may be activated downstream of the receptor. The demonstration that CD117 expression, pattern and extension in canine mammary tumours is correlated with proliferative activity may provide evidence for the utility of tyrosine kinase inhibitors in the therapy of neoplastic mammary disease.
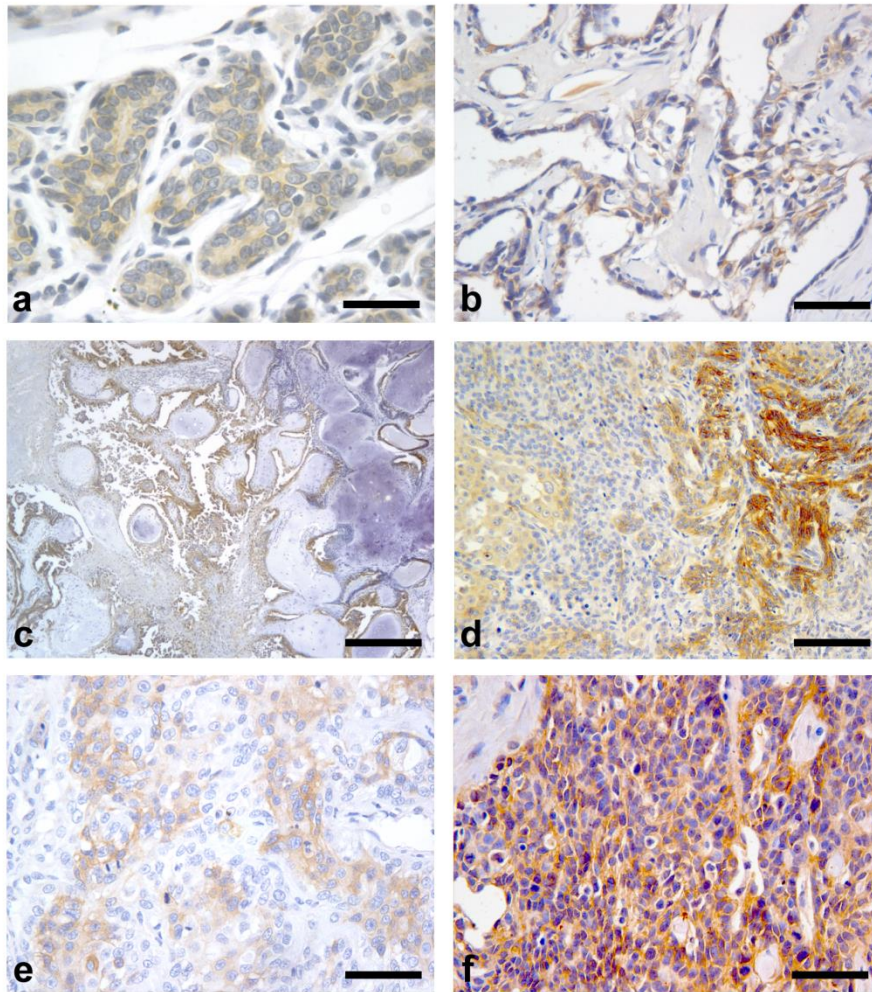
Figures

Fig. 1: Immunohistochemistry (IHC) of CD117 expression, pattern and extension.
A) Dog, normal mammary gland. Normal mammary ducts and lobules show a cytoplasmic and membranous pattern and diffuse IHC CD117extension. Bar, 50µm. **b)** Dog, mammary gland, fibroadenoma. Epithelial cells show a membranous pattern and diffuse extension to IHC CD117. Bar, 100µm. **c)** Dog, mammary gland, carcinoma in benign mixed tumour. Evidence of membranous and cytoplasmic pattern and diffuse extension to IHC CD117 in the epithelial component of the tumour. The cartilaginous component is negative. Bar, 500 µm. **d)** Dog, mammary gland, solid carcinoma. The cells to the left with squamous metaplasia show cytoplasmic positivity to IHC CD117, while the spindle-shaped epithelial cells on the right express membranous positivity. Bar, 200 µm. **e)** Dog, mammary gland, solid carcinoma. The epithelial cells present a cytoplasmic and membranous pattern and intermediate extension to IHC CD117. Bar, 100 µm. **f)** Dog, mammary gland, solid carcinoma. The epithelial cells

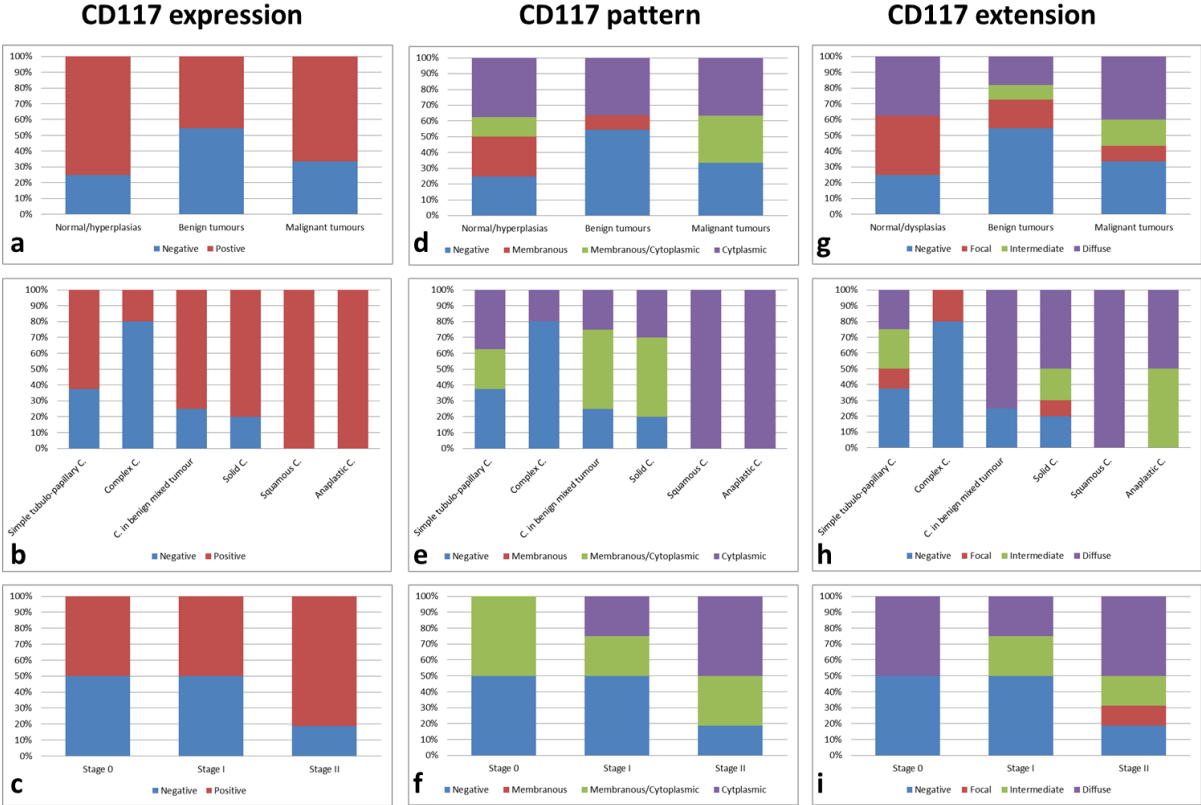present a membranous and cytoplasmic pattern and diffuse extension to IHC CD117. Bar, 100 μm.

Fig. 2: CD117 expression, pattern and extension.
CD117 expression (i.e. positive or negative immunohistochemical staining) in the 3 categories of tissues **(a)** in the histotypes of malignant tumours **(b)** and in the 3 histological stages **(c)**. CD117 pattern (i.e. the localization of the immunohistochemical stain as membranous and/or cytoplasmic) in the 3 categories of tissues **(d),** in the histotypes of malignant tumours **(e)** and in the 3 histological stages **(f).** CD117 extension (i.e. the amounts of tissue stained) in the 3 categories of tissues **(g),** in the histotypes of malignant tumours **(h)** and in the 3 histological stages **(i)**.
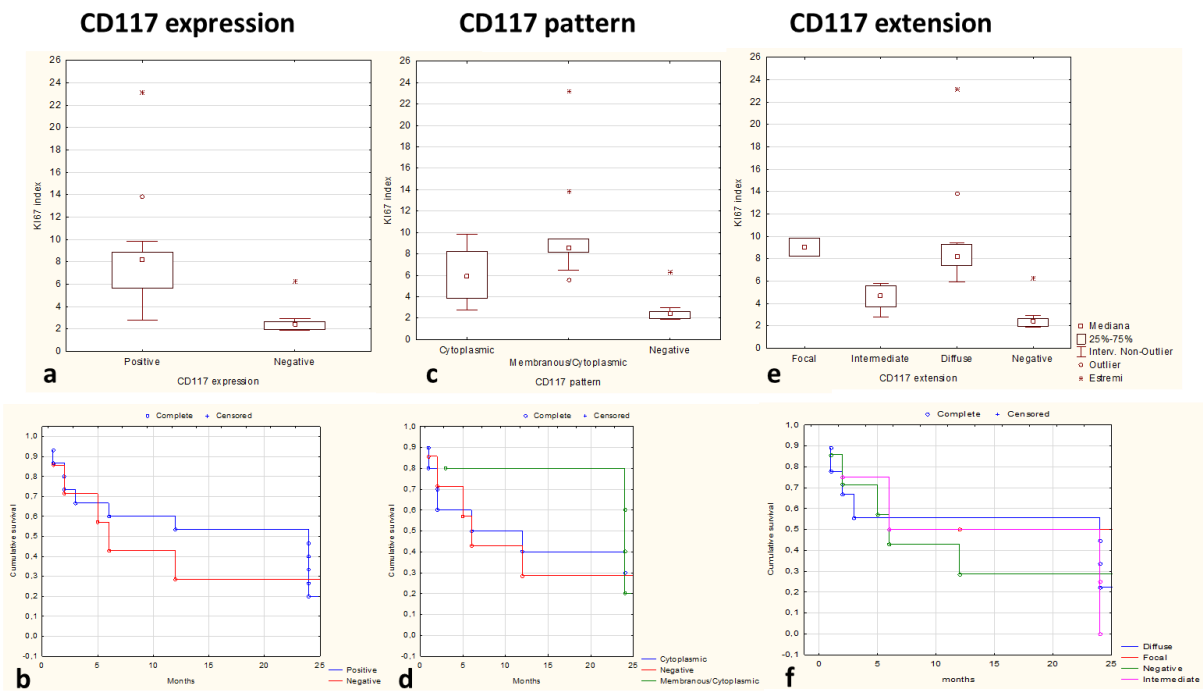
Fig. 3: Correlation between Ki67 index and CD117 expression, pattern, extension and survival rate.

Ki67 index of the malignant tumours in the two groups (positive; negative) of CD117 expression **(a)**, in the 3 groups (negative; membranous/cytoplasmic; C=cytoplasmic) of CD117 pattern **(b)** and in the 4 groups (negative, focal, intermediate, diffuse) of CD117 expression **(c)**. Kaplan-Meyer estimated curves in the two groups (positive; negative) of CD117 expression **(d)**, in the 3 groups (negative; membranous/cytoplasmic; cytoplasmic) of CD117 pattern **(e)** and in the 4 groups (negative, focal, intermediate, diffuse) of CD117 expression **(f)**.

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6. CONCLUSIONS

In Human Medicine, the discovery of molecular subtypes of breast cancer has produced further proofs of the fact that biological diversity consequently denies a unique therapeutic approach (Peppercorn *et al.*, 2008). The therapeutic approach to breast cancer varies according to the different molecular phenotypes and in the recent past it was highlighted the necessity to identify the molecular phenotypes also for the lymph node metastasis and eventual systemic metastases (Aitken *et al.*, 2010).

The information acquired from the research conducted in the past three years regarding the identification of molecular phenotypes in feline and canine mammary tumours revealed the importance of the phenotyping to fill current gaps regarding prognosis and a targeted therapeutic approach, since the primary tumour phenotype does not always overlap with that of its metastasis. The primary tumour phenotype assumes a predictive therapeutic role only in concordant cases, meaning that there should be a concomitant evaluation of both the primary tumour and its lymph node metastasis. Treatment planning based only on the primary tumour phenotype can lead to therapeutic failures if the lymph node metastatic phenotype differs from that of the primary tumour. To the best of our knowledge, this was the first report that identified molecular phenotypes in feline mammary tumours.

Regarding the correlation between primary mammary site phenotypes and the phenotypes of systemic metastases, the existence of both biological phenomena of concordance and discordance in metastatic sites has been confirmed. The prevalence of concordance between primary and metastatic sites supports the predictive therapeutic value of the primary tumour phenotype, minimizing any margin of error which can occur in rare discordant cases.

The research focused on various aspects of myoepithelial cells allowed to better characterized the four different myoepithelial cell morphological types in the normal and neoplastic

mammary gland using a panel of antibodies and confirmed the changes of myoepithelial cells towards mesenchyme from an myoepithelial to a mesenchymal phenotype.

The investigation that was conducted for the study of CD117 demonstrated that its expression, pattern and extension in canine mammary tumours is correlated with proliferative activity may provide evidence for the utility of tyrosine kinase inhibitors in the therapy of neoplastic mammary disease.

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7. OTHER PUBLICATIONS AND PROCEEDINGS FROM JANUARY 2011 TO JANUARY 2014

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