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# COMMON PROGNOSTIC BIOMARKERS IN CANINE MAMMARY TUMOURS - LITERATURE REVIEW AND RECENT PERSPECTIVES

## CEI MAI COMUNI BIOMARKERI DE PROGNOSTIC ÎN TUMORILE MAMARE LA CÂINE – RECENZIE A LITERATURII ȘI PERSPECTIVE RECENTE

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**ABSTRACT | REZUMAT**

Mammary tumours are one of the most frequent neoplastic diseases in canines and one of the leading causes of death in both dogs and humans. Canine mammary tumours (CMT) develop spontaneously and have high similarity with human breast cancer when it comes to several biological, clinicopathological, and molecular characteristics, including the detection of the same biomarkers. The evaluation of tumoral biomarkers is vital and enables better detection and diagnosis, the establishment of prognosis, and effective targeted therapy in cancer. This paper aims to review the most common and reliable biomarkers of canine mammary carcinomas based on current literature. Among the most promising biomarkers for CMT, micro RNAs (miRNAs) and mutations of the breast cancer 1 gene (BRCA1) and breast cancer 2 gene (BRCA2) are frequently mentioned. The most common and studied biomarkers of CMT, detected in both serum and tissue samples, are antigen Ki-67 (Ki-67), epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER-2), oestrogen receptor (ER), progesterone receptor (PR), and cyclooxygenase 2 (COX-2). Other useful biomarkers are tumour protein p53 (p53) and epithelial cadherin (E-cadherin). Despite many studies available so far in both human and canine mammary cancer, the detection of biomarkers remains uncommon, and their exact role is still unknown. Further detailed research needs to be done for a better understanding of mammary cancer pathology and for the development of new therapies and diagnostic tools.

**Keywords**: mammary tumours, dog, biomarker, oncology, prognostic

Tumorile mamare reprezintă unele dintre cele mai frecvente maladii neoplazice la câini și una dintre principalele cauze de deces, atât la câini, cât și la oameni. Tumorile mamare canine (canine mammary tumours - CMT) se dezvoltă spontan și au o asemănare mare cu cancerul de sân la om dacă se face referire la mai multe caracteristici biologice, clinico-patologice și moleculare, inclusiv detectarea acelorași biomarkeri. Evaluarea biomarkerilor tumorali este vitală și permite o mai bună detectare și diagnosticare a tumorilor, stabilirea prognosticului și o terapie țintită eficientă în cancer. Scopul acestei lucrări este de a revizui cei mai comuni și de încredere biomarkeri ai carcinoamelor mamare canine, pe baza literaturii actuale. Printre cei mai promițători biomarkeri pentru CMC, sunt menționați frecvent micro ARN (miARN) și mutațiile genei cancerului de sân 1 (BRCA1) și genei cancerului de sân 2 (BRCA2). Cei mai comuni și mai studiați biomarkeri ai CMT, detectați atât în ser, cât și în probele de țesut, sunt antigenul Ki-67 (Ki-67), receptorul factorului de creștere epidermic (EG FR), receptorul 2 al factorului de creștere epidermic uman (HER-2), receptorul de estrogen (ER), receptorul de progesteron (PR) și ciclooxigenaza 2 (COX-2). Alți biomarkeri utili sunt proteina tumorală p53 (p53) și caderina epitelială (E-cadherina). În ciuda multor studii disponibile până acum, atât în cancerul mamar uman cât și cel canin, detectarea biomarkerilor rămâne neobișnuită și rolul lor exact este încă necunoscut. Cercetări detaliate suplimentare trebuie făcute pentru o mai bună înțelegere a patologiei cancerului mamar și pentru dezvoltarea de noi terapii și instrumente de diagnosticare.

**Cuvinte cheie**: tumori mamare, câine, biomarker, oncologie, prognostic

Mammary tumours are among the most common types of neoplasia in domestic carnivores (32). More than 50% are diagnosed as malignant in canine patients and represent an important factor in dog mortality, which ranges from 20% to 55% due to high invasiveness and metastatic behaviour (49).

The incidence of canine mammary tumours (CMT) is greatly variable, depending on the geographic location, and is mostly influenced by age, breed, hormonal status, and also by genetic, nutritional, and environmental factors (47, 51, 53). CMT occurs mostly in female dogs, being extremely rare in males, and the mean age of diagnosis is usually 8 – 10 years old (4, 25), with unspayed and overweight bitches being at a

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higher risk (14). Regarding breed predisposition to CMT, statistics vary between countries in which epidemiological studies have been made, with no consensus reached until the present; however, most studies showed a higher incidence in pure breeds (51). Commonly encountered breeds are Samoyed, Miniature Poodles, Dachshunds, Labrador Retrievers, Maltese, Yorkshire Terriers, Cocker Spaniels, and German Shepherds (46). Regarding geographical location, information is variable depending on each country and the number of available studies. In Europe, a higher incidence rate of malignant mammary tumours was reported in the south, especially in countries where spaying is not routinely performed (51).

Currently, various methods are used in the treatment of CMT, with little information available regarding their efficiency. The prognosis of CMT relies on clinical stage and lymph node status, with additional lymphatic or vascular invasion and histological classification of the various subtypes of CMT on surgically removed masses according to WHO guidelines (21, 39).

The occurrence of spontaneous tumours in dogs and their similar clinical-biological, molecular, and pathological characteristics to human breast cancer make them suitable models for the study of tumorigenesis, aiming at the improvement of diagnostic tools and successful therapies (51).

Early diagnosis is very important in mammary carcinomas, and human medicine benefits from the use of the detection of tumour markers for prognosis and treatment.

Biomarkers are substances of mostly protein origin that can be detected and measured in all tissues, including neoplastic. They can give information about the presence and development of certain diseases and tumours, as well as the results of treatment and indicate the prognosis. In patients diagnosed with neoplastic diseases, cancer cells express specific proteins called tumour-associated antigens, which are referred to as biomarkers if detected in concentrations that are different from normal in other tissues, serum, or urine. Tumor markers can be characteristic of certain neoplastic cells or produced abnormally by them (53).

Determination of the same biomarkers as in human breast cancer could also be considered for dogs, as CMTs have similar immunohistochemical features and express the same substances (40). Determination of biomarkers in canine patients could greatly benefit the early diagnosis of malignant tumours, and the evaluation of tumour progression and the response to applied chemotherapy (18, 25). Still, due to their heterogeneous nature, the choice of the most appropriate biomarker remains a big challenge (25).

The purpose of this paper is to review the most common and reliable biomarkers of canine mammary carcinomas, with an emphasis on those that are easily detected by immunohistochemistry, for a better understanding and approach for both veterinary clinicians and pathologists.

To review the current veterinary literature describing prognostic markers in canine mammary tumours, we collected the necessary material by searching on PubMed (https://pubmed.ncbi.nlm.nih.gov/) and using the following keywords: "mammary tumors", "breast cancer", "canine", "literature review", "immunohistochemistry", "prognosis", "biomarkers", "gene", "expression". Selected articles were chosen based on addressing the most important and frequently used markers, their description and prognostic value, referring primarily to dogs and often with a brief comparison to human breast cancer for a better understanding of the process.

## OESTROGEN AND PROGESTERONE RECEPTORS

Oestrogen (ER) and progesterone (PR) are hormones necessary for the growth and regulation of functions of female reproductive organs, including the mammary gland; however, they also influence tumour development (39), thus being used in the molecular-based classification of mammary carcinomas in both human and veterinary medicine (26, 56).

In canine mammary tumours, healthy glandular tissue and benign lesions express both ER and PR. Oestrogen receptor alpha (ERα), which is one of two main types of oestrogen receptor, and also progesterone, have been found to have a lower expression in primary and metastatic mammary malignant tumours, with a worse prognosis for canine patients (11, 39). Comparing both expressions, the ER expression is commonly associated with a good prognosis and a lower stage, whereas the PR expression is commonly strongly associated with a longer postoperative survival time (30, 33). Kim et al. (2014) demonstrated in their study that an oestrogen receptor negative (ER-) and a progesterone receptor positive (PR+) tumour will have a poor outcome in comparison to a tumour with oestrogen and progesterone positive (ER+ and PR+); more importantly, tumours with ER- and PR- were considered to have the worst prognosis of all types (28). In their study, Brunetti et al. (2021) found that ER expression correlated better with survival compared to the histological grade (8).

However, the literature mentions few studies with increased levels of ER and PR in malignant mammary tumours in dogs (42). Also, at the moment, no consensus regarding the degree of ER expression has been reached, when taking into account the relation to prognosis (8).

In human breast cancer, the expression of ER, PR, and Human Epidermal Growth Factor Receptor 2 (HER

- 2) is evaluated to estimate staging and also improve the prognostic accuracy. High expressions of ER and PR indicate a better clinical outcome, while low expressions indicate a lack of sensitivity to endocrine therapy and a poor prognosis (25).

Currently, compared to human breast cancer, sex hormone-targeted therapy doesn't play an important role in CMT, with further studies needed to clarify their role in cancer treatment (25).

Although very useful in practice, routine evaluation of ER and PR is usually not applied in dogs, whereas in human medicine they are commonly used (8).

## EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR/HER 1)

EGFR belongs to the epidermal growth factor receptor (EGFR) family and is a transmembrane tyrosine kinase receptor that promotes angiogenesis, metastasis, and invasion in malignant tumours.

In human and veterinary medicine, this is associated with a poor prognosis. Studies show that EGFR is expressed in normal, hyperplastic, benign, and malignant mammary gland tumours, but its overexpression is associated with increased size of the mammary tumour, shorter survival time, tumour necrosis, relapse, metastasis, and a high mitotic index and histological grade (22). In one study from 2016(2), primary mammary carcinomas with and without lymph node metastasis were evaluated for EGFR expression. The authors found no significant difference in the overall survival of EGFR-positive or EGFR-negative canine patients, and the frequency of expression was not different when comparing primary tumours with lymph node metastasis and without metastasis.

Currently, EGFR inhibitors or a combination of EG FR and Cyclooxygenase-2(COX-2) inhibitors seem to be a good option for targeted treatment and control in advanced malignant CMT (22).

Even so, further studies are needed to establish this marker as an independent prognostic marker in both humans and dogs with mammary carcinomas (10).

## HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR 2 (HER-2)

HER-2 can be found in cell membranes and is a protein that belongs to the epidermal growth factor receptor (EGFR) family. It is considered an important prognostic marker for mammary carcinomas, being involved in the regulation of tumour growth, cell survival, differentiation, and proliferation, as well as migration and invasion (53). It is commonly used in human medicine. Overexpression of HER-2 has been associated with a lack of response to endocrine therapy and rapid tumour progression and metastasis, making it a marker for poor prognosis for humans and dogs (55). Ahern et al. (1996) obtained results attesting to the absence of HER-2 mRNA levels in benign tumours and their constant presence in malignant tissues (1). Kabir et al. (2017) attested to the amplification of HER-2, HER-3, and HER-4 in CMT cell lines (24). In one study on invasive carcinoma, tumour invasion was associated with HER-2 by the significant epidermal growth factor receptor (EGFR) and HER-2 mRNA expression in this tumour, compared to the in situ carcinoma (15).

In both CMT and human breast cancer, HER-2 is expressed in approximately 30% (25). In veterinary medicine, so far, positive correlations between serum and tissue expression of HER-2 have been observed, as well as HER-2 expression and histologically-assessed characteristics of malignancy, such as mitotic index and high histological grade, suggesting its role in carcinogenesis (48).

Despite its useful role in human medicine, for canine mammary tumours at the moment, there seems to be no agreement on the prognostic value of this marker, as the literature reveals contrasting results between studies. Some studies concluded that there is no difference between benign and malignant tumours regarding HER-2 expression (27, 45).

These variable results between studies and the high potential of this marker as seen in human medicine call for further studies to reach an agreement on the role of HER-2 as a prognostic marker in CMT.

## KI-67

KI-67 is a nuclear protein only detectable during the mitosis process and interphase, which makes it a marker for tumoral proliferation and apoptosis and one of the most studied in all cancer types, including mammary tumours.

Its presence can be detected by various types of techniques, including molecular biology. In veterinary and human pathology, it is routinely used in the evaluation of haematoxylin and eosin-stained slides from various neoplastic lesions, allowing for the determination of the mitotic index, but its precision is lower compared to the immunohistochemical evaluation, the latter providing the distinct expression of the marker in different phases of the cell cycle (41). Ki-67 can also be evaluated in cytological samples, providing an early diagnosis of mammary tumours and improved management of the case before excision (13).

Its evaluation as a serum biomarker in veterinary medicine needs further investigation, as only one study is available on dogs (36).

In human medicine, high expression of Ki-67 correlates with poor prognosis. Interestingly, patients

with high Ki-67 expression have a good response to chemotherapy (50).

In dogs, the expression of Ki-67 on immunohistochemistry is lower in benign tumours compared to malignant mammary tumours. High values have been observed in larger tumours, with inflammation and ulceration of the mammary gland, corresponding to a poorer prognosis, local invasion, metastasis to the lymph nodes, and overall poor survival (38). One study found a decreased expression of Ki-67 in older dog females compared to younger ones, indicating as a possible explanation the fact that tumour proliferation processes tend to be lower at an older age of the patient (38). Moreover, a high Ki67 index tends to correlate with p53 expression and is inversely correlated with ER positivity (8). In one study on canine mammary carcinomas, no significant difference in the frequency of Ki-67 between carcinomas with and without lymph node metastasis was noted, nor between the tumour and the metastasis, suggesting that the primary tumour and the paired lymph node metastasis are in general composed of cell populations with similar characteristics. In studies on human and canine mammary tumours, the association between the Ki67 index and the presence of lymph node metastasis remains controversial (2).

Even though it is the most frequently used biomarker for prognosis in mammary tumours, more studies are needed to fully assess its contribution to the adjuvant therapy of tumours. Nevertheless, in malignant tumours, the low expression of Ki-67 can be considered a favourable prognostic factor (53).

## TUMOUR PROTEIN P53

P53 is a protein that controls the cell cycle and apoptosis, and it is a suppressor of tumour development.

Mutations of the p53 gene are associated with tumour progression and are the most common genetic alterations in both human and canine mammary tumours (40).

In humans, mutations of p53 are very common, and its expression correlates positively with tumour-aggressive behaviour, a worse prognosis, and a shorter survival time (8).

In veterinary medicine, a high p53 expression is generally associated with poor overall survival, lymph node metastasis, a high histological grade, and recurrence (8), with two studies indicating a higher p53 expression in large-breed dogs, suggesting a possible breed predisposition (17, 29). In one study on 170 malignant mammary tumours in dogs (8), only 0.5% expressed p53; these tumours were labelled high-grade and with high proliferative activity, suggesting the involvement of the p53 gene in tumour progression of CMT. In one immunohistochemical study on p53 expression in CM, nuclear expression was only present in inflammatory carcinomas. Interestingly, tumours with lymphocyte infiltration and central necrosis tended not to express p53, while tumours with no central necrosis or lymphocyte infiltration expressed p53 (26).

Still, p53's role in tumour progression in animals remains unclear, due to the paucity of studies in veterinary medicine and the lack of determination of prognostic utility in other existing studies (34).

## CADHERIN-1 OR EPITHELIAL CADHERIN (E-CADHERIN)

E-cadherin is a transmembrane glycoprotein involved in epithelial cellular adhesion and is normally expressed in the cellular membrane of epithelial cells in normal mammary tissue (31).

The loss of E-cadherin expression in mammary tumours indicate an epithelial-mesenchymal transition (EMT), inducing mesenchymal characteristics in neoplastic epithelial cells, cell dissemination, migration, and invasion (20).

In both human and canine mammary tumours, low expression of E-cadherin on immunohistochemical examination is correlated with a worse prognosis, by increased size, malignancy and tumour progression, as well as histological grade, aggressiveness of metastases, and short overall survival (31). Asproni et al. (2015) identified that E-cadherin-positive dogs had a longer overall survival period and no lymphatic metastasis compared to those with loss of E-cadherin expression (3).

It is recommended to evaluate the expression of E-cadherin together with other biomarkers, the most common being Ki67 (25).

There is a paucity in the veterinary literature regarding E-cadherin expression in primary mammary carcinomas and lymph node metastasis. Recent studies (35) showed that E-cadherin expression in malignant mammary tumours is lower by 36.83% compared to benign tumours. Nevertheless, E-cadherin is a good diagnostic marker for mammary tumour malignancy and metastatic ability, and further studies should be made with an emphasis on the monitoring of the clinical course of the disease (55).

## CYCLOOXYGENASE-2 (COX-2)

COX-2 is an enzyme that plays a role in the catalysation of prostaglandin biosynthesis from arachnoid acid; perturbances of their metabolic processes lead to tumour progression, emphasizing the role of prostaglandins in tumoral development. The literature describes two isoforms: cyclooxygenase 1 (COX-1)

and cyclooxygenase 2 (COX-2). COX-1 is only expressed in normal tissues, while COX-2 is expressed only in inflammatory and neoplastic processes (43).

In humans with breast cancer, COX-2 overexpression is associated with advanced cancer stage, recurrence, the presence of metastases, and poor overall survival, and COX-2 inhibitors are used in chemotherapy (23).

In veterinary medicine, studies have demonstrated the presence of COX-2 expression in normal and neoplastic mammary tissues (7, 43), which is higher in malignant tumours than in benign ones (43). Araujo et al. (2016) showed in one of their studies that primary mammary carcinoma has high COX-2 expression when accompanied by lymph node metastasis, but no prognostic relevance has been found (2). Some studies identified elevated Cox2 expressions in association with increased angiogenesis and proliferation, as well as T-lymphocyte and macrophage infiltration of the tumour (10). One study (39) identified a close relationship between the more aggressive histological types of mammary tumours in dogs and high COX-2 expression. The same study reports no statistically significant relationship between COX-2 expression and the vascular and peritumoral invasion, the clinical stage, or the development of distant metastases. In both humans and dogs, selective COX-2 inhibitors are useful in the treatment of mammary carcinomas, by inducing dependent apoptosis, but combination with other antitumor drugs is strongly advised given the current state of knowledge on this marker.

## BREAST CANCER GENES 1 AND 2 (BRCA1 AND BRCA2)

Breast cancer genes 1 and 2 (BRCA1 and BRCA2) are genes coding a nuclear phosphoprotein that plays a role in the regulation of the cell cycle and the DNA damage response of mammary epithelial cells.

In human breast cancer, it is well known that these gene mutations are inherited and increase the possibility of developing mammary tumours, making them common markers for mammary neoplasia. Affected women have a risk of 56–84% of developing breast cancer (16).

So far, in veterinary medicine, BRCA 1 and BRCA2 are the most important susceptibility genes for CMT, for which the hereditary patterns are still under investigation (25). The loss of BCRA1 expression in dogs induces loss of apoptosis and tumorigenesis and is strongly associated with malignancy (37, 54). In one study (37) on canine mammary tumours, the BRCA1 expression on both nuclei and cytoplasm, compared to a solely nuclear one, proved characteristic of CMT, especially of the malignant types. Regarding BRCA2, no difference in expression between benign and malignant tumours has been noted (25), and one study described reduced expression of BRCA2 in mammary tumours in dogs compared to normal mammary tissues (54). Despite longtime studies on these two markers, the exact mutation mechanism of BRCA1 and BRCA2 and their decreased expression in tumoral tissue is still unclear in both human and veterinary medicine. Further studies focusing on the canine genome and its mutations are needed, for a better understanding of the process (53).

## MICRO RNA (MIRNA)

MiRNAs are small, non-coding molecules that play a role in the post-transcriptional negative regulation of gene expression. Literature shows their participation in the regulation of cell proliferation, differentiation, invasion, angiogenesis, migration, and apoptosis, making them true oncogenes or tumour suppressor genes (6, 9). In human medicine, miRNAs proved to have diagnostic and prognostic potential, with the prediction of therapeutic responses in breast cancer (5).

The CMT literature contains few analyses of the miRNA expression levels (6, 9, 52). Boggs et al. (2008) identified the same miRNA expression pattern in dogs as in humans for nine out of ten samples studied, suggesting its role in neoplastic processes in animals (6). Interestingly, different tumour types were shown to have different expression patterns of miRNA (12). Von Deetzen et al. (2014) described the fact that metastatic cells were different from those of the primary tumor in terms of miR-145, miR-143, miR-125a, miR-101, and miR-29b levels of expression (52). Recently, one study described that micro-RNA expression is different between metastatic and non-metastatic tumours, thus confirming its place among good metastasis biomarkers (9). Another study (19) identified that both miR-126 and miR-214 were significantly increased in dogs with malignant mammary tumours compared to healthy dogs. In vimentin-positive CMT, elevated levels of miR-21 have been observed, showing that miR-21 is produced from the mesenchymal cells, making it a possible more sensitive and non-invasive indicator for CMT (44). The evaluation of miRNA is relatively new but proves to be a promising and effective method of cancer diagnosis. Given the very similar expression pattern of dog miRNAs compared to human miRNAs, further studies are needed to be carried out on canine models, with miRNA-based drugs becoming more and more promising targeted therapies for mammary tumours (25).

## CONCLUSIONS

During the past few decades, many studies regarding mammary gland biomarkers have been made for humans and veterinary patients. Biomarkers are con-

sidered vital tools for the detection, diagnosis, prognosis, and treatment of cancer. The detection of the same biomarkers in both human and dog tumoral tissues marks the challenging yet infinite opportunities they bring to the development of this particular area in oncology. However, no productive conclusions have been drawn so far concerning their clinical application, and no ideal biomarker has been found thus, many papers recommend the assessment of two or more biomarkers at the same time, and combining clinical and pathological features and histological grades to obtain a more reliable diagnosis and prognosis. Currently, the most studied and reliable serum and tissue biomarkers for canine mammary tumours are Ki-67, EGFR, HER-2, ER, PR, and COX-2. Further detailed studies are needed to establish their definitive role in canine mammary gland carcinoma and to develop better treatment options.

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