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Review article

MOLECULAR MARKERS IN CANINE MAMMARY TUMORS

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Canine mammary tumors (CMTs) are one of the most common neoplasms in female dogs. Unfortunately, the current diagnosis often occurs in later stages, and there is a pressing need for more comprehensive data on treatment options to improve overall prognosis. Consequently, the early detection of these tumors is critical for improving treatment outcomes and survival rates. As such, biomarkers are essential for improving the diagnosis, treatment, and prognosis of CMT, the reason for which further research is required to enhance our understanding of the disease. The most studied biomarkers for CMT are evaluated from serum and tissue samples using different molecular approaches and relate to proliferation and cell cycle. Several biomarkers were also described regarding cell damage, autophagy and apoptotic-related pathways, hypoxia, angiogenesis, EMT, invasion, metastasis or cancer stem features. Overall, biomarkers have shown the potential to be used as a tool for the early detection of mammary tumors in dogs. However, more research is needed to validate these biomarkers and to develop sensitive and specific diagnostic tests. In this regard, we aimed to review known biomarkers and their role in CMT comprehensively. We also encouraged further investigations of reliable biomarkers that could improve treatment outcomes and survival rates for dogs with this disease.

Keywords: canine mammary tumors, molecular markers, coding genome, non-coding genome

INTRODUCTION

Canine mammary tumors (CMTs) are one of the most common types of cancer in female dogs. Among malignant tumors, tubular carcinoma (adenocarcinoma) is the most predominant, followed by papillary, solid, complex, and carcinosarcoma [1]. Conversely, benign tumors are classified as fibroadenomas, ductal papillomas, benign mixed tumors and simple adenomas [1]. Treatment for mammary tumors in dogs typically involves surgical removal of the affected gland, followed by chemotherapy or

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radiation therapy if the tumor is malignant. In some cases, a combination of surgery, chemotherapy, and radiation may be recommended, but are highly costly [1-3].

Factors, including age, breed, reproductive status, and hormonal influences, influence the risk of developing CMTs [4]. Intact female dogs have a higher risk of developing mammary tumors than spayed dogs, and the risk increases with age [2,4]. Dachshunds, Cocker spaniels, Miniature Poodles, Yorkshire Terriers, German shepherds, and mixed-breed dogs have been described to have an enhanced prevalence of mammary neoplasia [5]. CMTs also exhibit the pathologic characteristics of human breast cancers, the invasive ductal carcinoma being the primary tumor type, followed by carcinoma *in situ*. The lymphatic invasion, regional lymph node metastasis, and the mitotic index can also be used to characterize CMT [6]. No specific mutational patterns were identified until the present [1].

CMTs are characterized by variable biological features, such as tumor size, histological grade, growth mode, and lymph node status, hamper individual clinical outcomes estimation. However, these clinical and histological factors are only sometimes used in CMT management [7]. A molecular approach to biomarkers exploration has become more popular, as tumor markers analysis is a valuable tool in oncology procedures [8-10]. This investigative method is less invasive, non-complex, and inexpensive, increasing the interest for further research. A growing number of studies have investigated prognostic and predictive markers of mammary tumors development in canine models, such as hormone receptors, proliferation and apoptotic markers, markers of angiogenesis and metastasis, or oncogenes [2,11].

In 2018, the knowledge of CMT biomarkers was summarized in a comprehensive review by Kaszak et al. [1]. The most reliable biomarkers were described depending on cellular pathways in which they are prevalently involved: Ki-67, PCNA, HER-2, and p53 as biomarkers of cancer cell proliferation and apoptosis; E-cadherin, CEA, and CA15-3 as biomarkers of metastatic potential; VEGF, EGFR, as biomarkers of angiogenesis; COX-2 as a biomarker of inflammation in the cytoplasm, and hormone receptors on the cell surface. In this review, we provide a better understanding of the progress of predictive biomarkers validation in CMTs and contoured the future directions of research. Additionally, we describe non-coding genes and the specific types of cells, such as cancer stem cells (CSCs) and circulating tumor cells, related to CMTs, whose role should not be underestimated.

Despite the considerable prognostic potential of some markers at the testing level, only a few of them are used in the management of CMT. The reasons arise from insufficient data upon prospective multivariable survival studies and low established sensitivity and specificity. In this regard, further investigations of CMT prognostic and predictive markers of CMTs are encouraged to ensure a faster diagnosis, providing better options for an appropriate therapeutic decision and developing new agents against novel therapeutic targets.

Canine mammary tumor markers involved in cell cycle and proliferation

A fundamental and most recognizable hallmark of cancer, including breast cancer, is sustaining proliferative signaling, which comprises dysregulation of the cell cycle [12]. Certain molecular factors are distinctively regulated during this process and, therefore, can potentially be used as biomarkers of a particular cancer type and subtype, stage, or dysregulated pathway [12-14].

Several proliferation markers can be used to assess the growth rate of CMTs. The most studied proliferation marker is represented by the Ki-6 or MKI67 (a marker of proliferation *Ki-67)*. Ki-67 is a protein that is expressed in proliferating cells during various phases of the cell cycle. It is commonly used as a proliferation marker in breast cancer, including CMTs. Ki-67 is a nuclear, non-histone protein that can be differentially detected through distinct cell cycle phases [13]. Specifically, levels of Ki-67 protein were correlated with G1, S, G2 and mitosis (active stages of the cell cycle), markedly detectable in the last one and absent in quiescent cells in the G0 phase. The protein seems to act as a surfactant and stabilize the chromosomes in the mitotic phases after the nuclear membrane disassembling, assisting the proper chromosome interaction with the mitotic spindle and the intracellular compartmentalization [15]. This explains why Ki-67 is localized in the nucleus during the interphase, while in mitosis, it is mainly detected on the chromosomes' surface. However, some evidence suggests that Ki-67 can be detected to a certain degree at the ribosomal RNA transcription area, regulating its synthesis [13,15-17].

Although Ki-67 is investigated as a tissue marker of malignant proliferation, analysis of this protein level in serum samples can represent an additional potential non-invasive assessment. Neumann and colleagues aimed to determine whether serum levels of Ki-67 are increased in malignant cases. Thereon, Ki-67 was measured in three clinically distinct dog groups. Results showed a positive correlation between protein level and degree of malignancy, depicting significantly increased serum concentrations in dogs with malignancies, including mammary carcinoma. Also, low concentrations were identified in dogs with nonmalignant diseases, including mammary gland adenoma, while detectable Ki-67 levels were not found in healthy dogs [18].

Similarly, in a more recent study, serum levels of Ki-67 were evaluated in healthy dogs, non-neoplastic diseased dogs, and dogs with various malignant and benign tumors. Results showed that significantly high serum Ki-67 levels were detected in dogs with various malignant tumors (carcinomas, sarcomas, or lymphomas) compared to healthy and benign diseased dogs. However, there were no differences in the marker concentration between mammary adenocarcinoma and adenoma and malignant subtypes, such as carcinomas, sarcomas, and lymphomas [13].

Proliferating cell nuclear antigen (PCNA) is another protein expressed in proliferating cells. It plays a vital role in DNA replication and repair, commonly used as a proliferation marker in many types of cancer, including CMTs [19]. PCNA *is used to* discriminate different phases of cell division. In contrast with Ki-67, the highest expression level

of PCNA is attested in the S phase of the cell cycle, which continues to persist during mitosis due to the long half-life of the marker [20]. Nevertheless, there is evidence that discriminates against the specificity of the marker, as it is also involved in complex processes of DNA repair in a proliferative-independent way.

PCNA and Ki-67 markers were some of the earliest markers of prognostic significance in canine mammary malignancies [20-22] and continue to be evaluated in combination with other prognostic markers for better results. A multivariate survival analysis revealed higher proliferation indexes of Ki-67 and PCNA in malignant tumors compared to benign and adjacent non-neoplastic mammary glands [14]. Furthermore, adjacent non-neoplastic mammary glands found the lowest proliferation indexes (0-24% for Ki-67 and 12-47% for PCNA). Also, there was a link between the proliferation index of intratumoral Ki-67 and tumoral PCNA, and identified clinicopathological factors (histological grade of malignancy, metastatic lymph nodes, mitotic index and histological type) [23].

Cyclins and cell cycle regulation signaling were the top activated canonical pathways in CMTs linked to pathogenesis, revealed by a bioinformatic study on CMT tumors [24]. Cyclins are regulatory proteins of cell division, regulating the progression of the cell through the proliferation phases and modulating the transition of the cell through cell cycle checkpoints together with cyclin-dependent kinases (CDKs) [25]. Cyclin E/CDK2 and Cyclin D/CDK4-6 are complexes that regulate the transition from G1 to the S phase; hence, their overexpression is evaluated as a biomarker of malignant cellular growth, including breast cancer. In contrast, other cell cycle regulators, such as p21, p27 and p53, are known to be downregulated and are also investigated in the targeted cancer therapy [26-29].

Cyclin D1 is a protein that regulates the cell cycle and is often overexpressed in breast cancer [30]. It is also commonly used as a proliferation marker in CMTs being overexpressed in both benign and malignant CMTs, suggesting that it may play a role in the early stages of tumor development [31]. Another study found that cyclin E was associated with a poor prognosis in dogs with malignant CMTs, indicating that it may be a potential therapeutic target [31-33].

Mini-chromosome maintenance (MCM) proteins are a group of highly conserved proteins that play an essential role in DNA replication. These proteins initiate DNA replication and unwind DNA at the replication fork. Studies have shown that aberrant expression of MCM proteins is associated with various types of cancer, including canine mammary tumors [34]. Although some *in silico* and immunohistochemical analyses suggested its potential as a valuable prognostic biomarker of proliferation in breast cancer [20,35,36], up to our knowledge in the last ten years, there have been no studies on MCM regulation in CMT. Only one article in the literature described the correlation between MCM-3 expression levels and grade of malignancy. It indicated the detection of MCM-3 protein in 70% of mammary adenocarcinoma cases and underlined a positive correlation between increased marker expression level and high grade of malignancy [34].

Like MCM, investigations of cyclins' potential as prognostic biomarkers in canine mammary malignancy have not acquired a scientific interest in the last period. According to immunohistochemical analysis, positive staining for cyclin A was measured in half of the canine malignant mammary tumor cases and was not identified in benign mammary tumors. Limited expression of cyclin D1 was registered [37]. Another study presented 60% of Cyclin D expression level in pre-cancerous lesions and 44% of cancerous lesions of the canine mammary gland [31].

Human epidermal growth factor receptor 2 (HER2 or ErbB-2) is a protooncogene that encodes a glycoprotein responsible for increased cell proliferation and differentiation [38]. HER2 overexpression was related to an increased number of HER2 gene copies (and overall survival, but no correlation with the expression levels for the ER, Ki-67, grade, metastasis, and tumor-specific survival [39].

High expression of HER-2, Cox-2, and Ki67 found in primary mammary carcinomas was correlated with increased expression of these markers in paired lymph node metastasis. Moreover, a shorter overall survival was associated with a Ki-67 index higher than 24%, plus a large tumor size and extracapsular extension in the lymph node metastasis [40]. Representative CMT markers with a role in cell cycle modulation are shown in Figure 1.

Similarly, complexes CDK2-Cyclin E and CDK1-Cyclin A are upregulated during the S and G2 phases. M phase represents a phase of cell division when intracellular components with genetic material are equally distributed between new daughter cells. During this phase, mitotic cyclin B forms a molecular complex with CDK1, further implied in the phosphorylation of target substrates and reorganization of cell structure. CMT markers, like Ki67, PCNA and MCM3, are found to be implicated in the regulation of Cyclin-CDKs complexes level during cell proliferation.

CMT markers involved in cell damage, autophagy and apoptotic-related pathways

Besides sustaining proliferation, resisting cell death constitutes another essential feature of tumorous cells. There are different types of cellular death, among which apoptosis attracts much scientific interest as it comprises important intra – and extracellular pathways with a critical role in regulating the cellular death rate. Apoptosis represents programmed cell death where malfunctioning or potentially damaged cells are removed to ensure a healthy status [12,43]. Another type of cellular death is defined by autophagy. Unlike apoptosis, autophagy is usually triggered by extracellular stressors, such as nutrient deprivation, and constitutes a process of self-degradation by the cells' lysosomal system. It was brought to the attention that stimulation of autophagic pathways can exert a dual role in the fate of cancer development, being either an appealing therapeutic strategy in the phases of cancer initiation or a tumour's ally by supporting outliving and preventing the accumulation of unnecessary components [44,45].

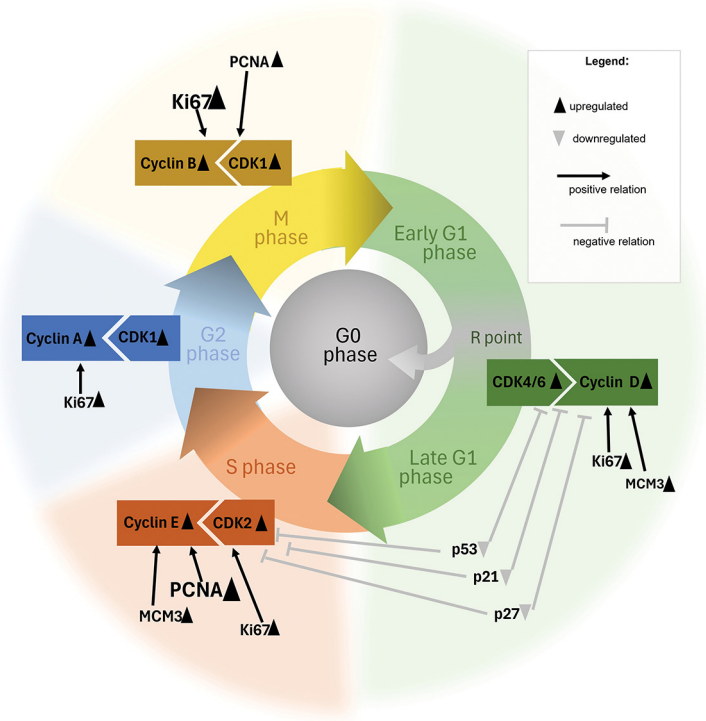

Figure 1. Cell cycle and the most representative markers in CMT. The cell cycle can be divided into four main phases: G1, S, G2, and M. Particular molecular reactions occur during each phase, allowing the cell to increase constantly until it temporarily or permanently enters the G0, a quiescent phase. Entering the next proliferation phase is strictly controlled by the levels of cyclins and cyclin-dependent kinases (CDKs) and other regulatory proteins, which, in turn, regulate Cyclin-CDKs complexes. Overexpression of the CDK4/6-Cyclin D complex during the G1 phase allows cell progression to the S phase and DNA synthesis. Also, during this phase, tumor suppression proteins, such as p21, p27 and p53, are found to be downregulated and have a significant role in the regulation of Cyclin-CDKs complexes.

P53 is one of the most critical tumor suppressor proteins and is usually downregulated in cancers, including breast cancer [38]. Its role is to ensure genetic stability and appropriate non-mutant cell division. The goal can be achieved through many processes, such as regulation of DNA repair, cell-cycle arrest, transactivation of numerous target genes, or triggering the activation of apoptotic or autophagic pathways. As such, some dysregulated apoptotic or autophagic key factors become potential markers for the signature of an abnormal survival rate of the cancerous cells. Therefore, TP53 protein modulates multiple cell death-associated molecules, such as Bim, NOXA, PUMA, PTEN, AMPK, Bcl-2, and Bax, the last two being of increased importance as Bax/Bcl-2 ratio plays a crucial role in determining the outcome. Consequently, this protein is called "The Guardian of the Genome" [46,47]. Mechanisms, though TP53 regulates apoptosis, are schematically represented in Figure 2.

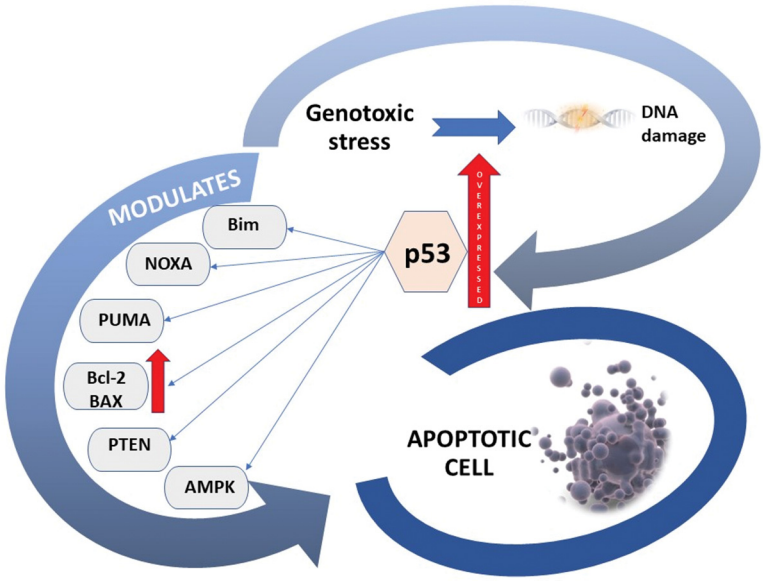

Figure 2: "The Guardian of the Genome" – Apoptosis. Protein p53 is a tumor suppressor protein that significantly prevents abnormal cell proliferation if genetic aberrations occur. It is also called "The Guardian of the Genome". Several considerable functions in which p53 is involved are as follows: 1. P53 is actively implied in the process of DNA repair when genotoxic stress of any nature occurs. In this case, p53 upregulation favors the activation of DNA repair genes, therefore abolishing the risk of further cell proliferation with damaged genetic material in the first place. 2. P53 also modulates the expression level of several pro-apoptotic proteins such as Bax, Bim, NOXA, PUMA, PTEN and AMPK. It upregulates the Bax/Bcl-2 ratio, mainly leading to the activation of the intrinsic apoptotic pathway.

A study aiming to analyze proliferation, differentiation, and apoptosis in canine breast cancer showed significantly altered expression of p53 and Bcl-2 in canine mammary tumors compared to corresponding adjacent tissue. Increased nuclear localization of the p53 protein and cytoplasmic localization of Bcl-2 were supported by immunohistochemical analysis. Although in the literature, the downregulated profile of p53 protein is usually associated with rapid malignant evolution and bad prognosis, it mainly refers to a wild type of protein. In contrast, increased expression of the mutant type of p53 presented in this study has been broadly connected with the positive evolution of the disease. These expression features, with additionally assessed upregulated PCNA and estrogen receptor (ER) and downregulated levels of cytokeratin, could indicate apoptosis-resistant phenotype, loss of differentiation potential, and increased proliferation rate [42].

Another study that included different types of canine mammary pathology (tubulopapillary carcinoma, complex carcinoma and carcinosarcoma) evaluated the expression of anti-apoptotic protein Bcl-2 and pro-apoptotic cysteine proteases caspases 3, 8, and 9. Differences in the expression level of Bcl-2 were statistically significant according to tumor types, being moderate to strongly expressed in carcinosarcoma

cases compared to tubulopapillary and complex carcinoma cases. In contrast, caspases showed no statistical significance. Additionally, the immunohistochemical analysis of PCNA and Ki-67 presented heterogeneous staining throughout different cases [41].

Raduly et al. [48] discussed an interesting approach: They investigated ABT-199, a small molecule known for selectively inhibiting Bcl-2. The research demonstrated that ABT-199, when applied to human and canine breast cancer cell lines, induced inhibitory effects on cell viability. Compared to untreated cells, 98.60% of necrotic cells were assessed in the P114 canine cell line treated with ABT-199. Suppressor mechanisms are also confirmed by assessment of apoptosis, cell cycle disruption, and migration inhibition.

In tissue samples obtained from dogs with simple adenomas, adenocarcinomas of the mammary gland and lymph node metastases with normal mammary glands, cyclin-dependent kinase inhibitors, such as p21 and p27, were investigated. Analysis of mRNA level demonstrated a lifted expression degree of p21 in adenocarcinomas, whereas in adenomas and metastases, the expression level was heterogeneous. Additionally, an overexpressed protein level was found in metastases samples compared to samples of adenocarcinomas. Interestingly, levels of p27 followed the same expression pattern in adenomas and metastases samples, but in contrast with p21, expression pf p27 was diminished in the adenocarcinoma samples. The expression level of p53 was heterogeneous in adenomas, adenocarcinomas, and lymph node metastases and different from one canine patient to another [33].

CMT markers involved hypoxia, angiogenesis, EMT, invasion and metastasis

Hypoxia is an important mechanism that was proven to be related to multiple pro-angiogenic pathways, increasing genomic instability, and inducing resistance to chemotherapy and radiation therapy [49]. A strong interaction between tumor hypoxia and tumor metabolism has been identified [50]. In CMT, hypoxia is associated with a more aggressive tumor phenotype and a higher risk of metastasis [51]. Hypoxia-inducible factor 1 alpha (HIF-1α) is a crucial transcription factor that regulates the cellular response to hypoxia. HIF-1α is overexpressed in CMT, and its expression has been associated with tumor grade, size, and lymph node metastasis [52,53]. Also, the serum level was higher in dogs with tumor metastasis and recurrence [53]. In addition, hypoxia has been linked to the activation of signaling pathways that promote tumor cell survival and proliferation, such as the PI3K/Akt and MAPK pathways. Hypoxia can also induce gene expression in invasion and metastasis, such as matrix metalloproteinases (MMPs) [51,54]. The role of epithelial-mesenchymal transition (EMT) and *angiogenesis* is significant in controlling tumor growth and *metastasis* [55]. A unique characteristic of EMT is represented by the loss of E-cadherin, which can stimulate metastasis through increasing capacity to migrate and invade the adjacent microenvironment [56]. Immunohistochemical methods in primary canine mammary

carcinomas found an interconnection between E-cadherin+/vimentin+ cells and higher tumor grade, suggesting that EMT plays a crucial role in the metastasis of CMT [57]. In vitro and in vivo studies performed on metastatic CMT cell line CF41, respectively, on canine mammary cancer xenograft model, proved that TGF-β1 silencing in combination with metformin treatment suppressed the process of EMT and potential metastatic [58]. ZEB1 and ZEB2 are vital genes and transcription factors related to EMT presented as therapeutic targets in CMTs [59]. Another study revealed SNAIL, TWIST, and ZEB mRNA to have decreased expression levels in tumors than in healthy tissues [60].

It is recognized that angiogenesis is vital in tumor growth, invasion and metastatic process [61]. VEGF-B and EGFR genes were overexpressed in mammary gland carcinomas compared to adjacent tissues [62]. Studies performed on 248 formalin-fixed paraffin-embedded CMT samples demonstrated that the VEGFR deregulation can promote vasculogenic mimicry formation [63]. EGFR, COX-2, and VEGF overexpression were related to increased angiogenesis and invasion in the malignant CMT [64-66]. Aspirin was found to inhibit the migration and proliferation of canine mammary gland tumor cells in a dose and time-dependent manner; the inhibition was mediated by Wnt signaling pathways and by regulating MMPs and EMT [67]. CMTU309 carcinoma cells exhibited high contractile forces; furthermore, there has been an interdependence between the high contractility, 3D-invasion potential and the activity of 5'AMP-activated protein kinase (AMPK)[68].

Galectin-3 is a carbohydrate-binding protein that has been demonstrated to be involved in different steps of metastasis, such as cell proliferation, cell-cell and cell-ECM adhesion, and stimulation of angiogenesis [69]. Galectin-3 expression was up-regulated in CMT-U27 cells exposed to hypoxia; moreover, *in vivo* studies identified overexpression of galectin-3 in hypoxic necrosis-surrounding areas [31]. Vascularization, lymph node involvement and metastasis were found to be responsible for high serum levels of VEGF in female dogs with breast cancer-promoting tumor progression [53].

Microarray analysis identified 265 genes related to human breast cancer from 1011 significantly differentially expressed genes in metastatic CMTs compared with non-metastatic carcinomas [70]. In primary and metastatic canine mammary gland tumor cell lines, hypoxia-induced downregulation of HIF1α via downregulation of TNF-stimulated gene-6 (TSG-6) reduces cancer cells' metastatic and proliferative abilities [51]. Tumor cell growth can be stimulated by IL-6, and IL-8, promoting loco-regional relapse and metastasis in CMTs [71]. Fucoxanthin, a carotenoid extracted from brown algae, exerted anti-angiogenic activity on CMT-U27 cells by increasing the angiopoietin 2 (Ang2) expression [72]. Treatment with three selenium compounds (sodium selenite (SSE), methylseleninic acid (MSA), and methyl selenocysteine (MSC)) induced cell apoptosis in the CTM1211 cell line and caused downregulation of the VEGF-alpha, Ang-2, and HIF-1 alpha expression and upregulation of PTEN expression, and therefore inhibition of angiogenesis [73]. A study performed on 70 canine mammary

tumors (28 benign and 42 malignant) identified over-expression of COX-2 and VEGF that may promote the increase of angiogenesis and tumor growth [73]. An increase in microvessel density and Cox-2 expression was correlated with a worse prognosis and decreased overall survival [74]. Overexpression of EGFR was associated with an increase in tumor growth, an increase in metastatic capacity and histological grade of tumor malignancy, and poor overall survival [66,75].

Proliferation, dedifferentiation and loss of cell-cell contacts are the main signs of the activation of the metastatic cascade [76]. Therefore, genes related to epithelial differentiation (EGF, EGFR, MAP2K6, STAT 5), cellular adhesion (CLDN5, CTNNAL1, MUC1, PECAM1), membrane receptors (EGFR, FGFR1, GHR, PDGFR, TGFBR, TIE1) and angiogenesis (ANGPT 2, ANGPTL1-4, FIGF, TIE1) were observed to be underexpressed including in CMTs [76]. A summarized representation of potential prognostic markers with regulatory role in aforementioned cellular pathways in CMTs is depicted in Figure 3.

Markers involved in cancer stem cell regulation.

In malignant tumors cancer stem cells (CSCs) represent a small subpopulation of cells with unique features that enable them to self-renew, differentiate and resist chemo – and radiotherapy [80,81]. These cells are involved in the initiation, recurrence, and metastasis [81]. For these rationales comprehension, the properties of CSCs have the highest significance [82]. Using bioinformatics approaches, an embryonic stem cell gene expression signature was identified in CMT [83].

The morphological differentiation of cultured canine cancer stem cells from cancer epithelial cell lines into endothelial-like cells supports the vasculogenic mimicry phenomenon from an ultrastructural point of view [84].

Proteosomes, stress inducers, and mammalian targets of rapamycin (mTOR) within signaling pathways are used as potential therapeutic targets for cancer stem cells [OBJ].mTOR inhibitors, such as everolimus and temsirolimus, showed dose-dependent responses for decreasing cell viability in sphere-forming and adherent cells [82]. In a recent study, canine mammary carcinoma cells showing CSC features resisted doxorubicin, but were highly sensitive to metformin [85].

CSCs, isolated from REM 134 cell, line were found to possess an increased ability to form tumorospheres; moreover, they expressed embryonic stem cell markers and were found to be relatively resistant to the cytotoxic effects of common chemotherapy and radiation. Also, TGFβ treatment increased invasiveness correlated with the induction of an EMT [86]. Salinomycin inhibited sphere-formation and invasive capacity in canine mammary CSCs in a dose-dependent manner; the expression of Wnt/β-catenin signaling-associated proteins was downregulated after treatment in the spheres [87].

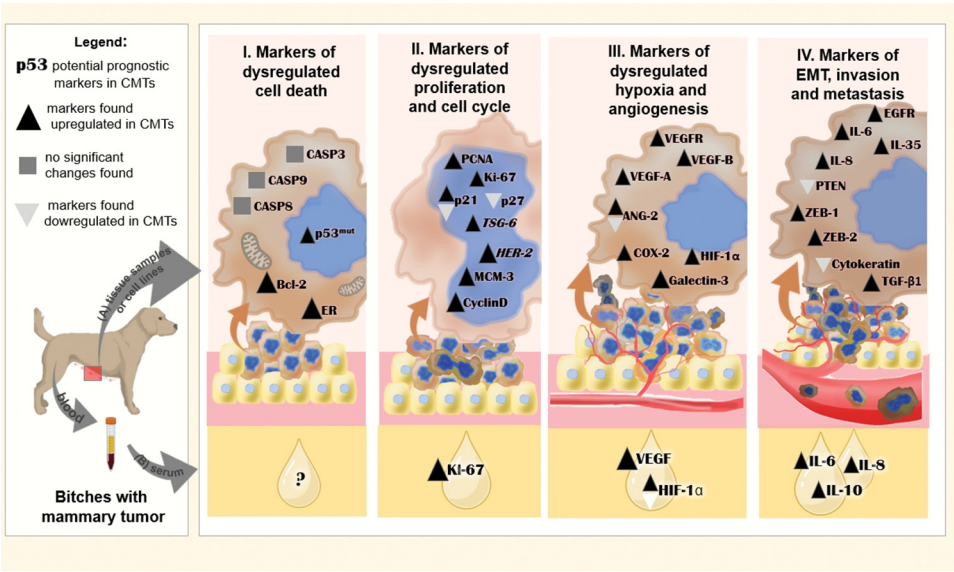

Figure 3. Graphical representation of potential prognostic markers for different stages of CMTs found either in tissue samples or cell lines (A) or in the serum (B). **I. Markers of dysregulated cell death.** The most recognizable markers are those related to malfunctioning apoptotic pathways and have dysregulated apoptotic key factors, such as p53 and Bcl-2, and protein effectors, such as Casp3, 8 and 9. **II. Markers of dysregulated proliferation and cell cycle.** Markers Ki-67 and PCNA attest to cell proliferation at different cell cycle phases. Abnormal expression of cyclins and cyclin-dependent kinase inhibitors, such as Cyclin D, p21, and p27, can also carry a significant prognostic role. **III. Markers of dysregulated hypoxia and angiogenesis: Si**milar to hallmarks attested in human breast cancer, overexpression of VEGF, VEGF, and HIF-1a indicates an increase in angiogenetic processes. Signs of potential promotion of new blood vessel spouting have been suggested by the unappropriated degree of other mediators, such as ANG-2, COX-2, and galectin-3. **IV. Markers of EMT, invasion and metastasis.** The last stage of cancer development represents invasion and metastasis, implying many changes in the tumor microenvironment. As such, enhanced signaling activities of particular cytokines, such as IL-6, IL-8, IL-10 and IL-35, participate in inflammation and tumor progression. The abnormal cellular concentration of some markers, such as ZEB-1, ZEB-2, and cytokeratin, can be related to the advantage of EMT. Note that markers depicted with both upregulation (▲) and downregulation (▼) marks denote controversial results according to different studies or can have different expression levels according to distinct types of CMTs, for example, as in malignant tumors versus metastasis (see in the description in the main text for particular marker expression). **Abbreviations:** ANG-2 – Angiopoietin-2; Bcl-2 – B cell lymphoma protein; CASP3 – caspase 3;CASP8 – caspase 8; CASP9 – caspase 9; COX-2 – cyclooxygenase-2; EGFR – Epidermal growth factor receptor; ER – estrogen receptors; HER2 – Human epidermal growth factor receptor-2 gene; HIF-1α – Hypoxia Inducible Factor 1 Subunit Alpha; IL-10 – interleukin 10; IL-35 – interleukin 35; IL-6 – interleukin 6; IL-8 – interleukin 8; Ki-67 – Proliferation Marker Protein Ki-67; MCM3 – minichromosome maintenance 3; P27 (CDKN1A) – Cyclin Dependent Kinase Inhibitor 1A; P27 (CDKN1B) – Cyclin Dependent Kinase Inhibitor 1B; $p53^{mu}$ – mutant tumor suppressor protein p53; PCNA – Proliferating Cell Nuclear Antigen; PTEN – Phosphatase And Tensin Homolog; TGF-β1 – Transforming Growth Factor Beta 1; TSG-6 – TNF-stimulated gene-6; VEGF-A – Vascular endothelial growth factor A; VEGF-B – Vascular endothelial growth factor B; VEGFR – VEGF receptor; VEGFR – Vascular endothelial growth factor receptor; ZEB-1 – Zinc Finger E-Box Binding Homeobox 1; ZEB-2 – Zinc Finger E-Box Binding Homeobox 2.

Altered non-coding RNAs in CMT

Several common microRNAs and lncRNAs were identified in CMTs and human breast cancer [88,89]. miRNA expression patterns in CMT showed essential differences between the tumor and normal tissue and between the metastatic and non-metastatic cases [88]. An RNA-seq study evaluating the alterations of mRNA levels in CMT versus adjacent normal tissue revealed 503 altered transcripts [90]. A recent study showed that six secretory exosomal miRNAs (miR-26b, miR-9, miR-1306, miR-1841, miR-132 and miR-345), isolated from different canine cancer cell lines, had changed levels in canine tumor [91]. Other studies present some serum candidate biomarkers (miR-181a, miR-34c, miR-18a, miR-19b, miR-122, miR-29b and miR-125a) linked to some clinical characteristics, such as grade, metastasis, and survival [92].

A recent study revealed that tumors positive for vimentin staining were correlated with increased miR-21 levels, indicating that this transcript is released from cells with mesenchymal features [93]. The results of the study by Ren et al. showed that miR-124 is an important tumor suppressor capable of inhibiting CMT cell proliferation, migration and invasion by targeting the CDH2 [94]. miR-497 was positively correlated with p63 and PTEN, critical genes related to breast cancer; moreover, miR-497 was proved to have the capacity to regulate apoptosis via the IRAK2/NF-$\varkappa$B axis in the CMT cells [85].

Long noncoding RNAs (lncRNAs) are described as RNA transcripts longer than 200 nucleotides unrelated to the protein-coding features [89]. Genome-wide investigation discovered many lncRNAs in different cancers characterized by tumor progression and metastasis. Also, lncRNAs, including CMTs, were identified as promising novel biomarkers and therapeutic targets for cancer [89]. The online tool FEELnc, comprising 20 canine RNA-seq samples, expands the canine genome annotation to include 10374 lncRNAs and 58640 mRNA transcripts [95]. A study by Le Béguec et al., 2018, presented an extended repertoire of more than 10000 lncRNAs in the domestic dog [95,96]. In this regard, two lncRNAs (lnc40589 and lncRNA34977) with different behavior have been identified in CMT cells. Specifically, lnc40589 inhibited the cells' proliferation, invasion and migration, while the opposite effect was registered for lncRNA34977; furthermore, lncRNA34977 promoted mammary tumorigenesis in mice [89]. In another study, tamoxifen sensitivity and tumor development were affected by lncRNA-42060 through the regulation of the miR-204-5p/SOX4 axis in CMTs [97]

Table 1. Relevant examples of cell proliferation biomarkers with implications in CMT management

Animal models/ pathology	Biological sample	Marker	Expression profile	Observations	Ref.
Healthy dogs (19 cases), non-neoplastic diseased dogs (26 cases) and dogs with various malignant tumors (carcinomas:35, sarcomas:26, lymphomas:21)	Serum samples	Ki-67	Upregulated in malignant tumors versus healthy dogs	No differences of the marker concentration between mammary adenocarcinoma and adenoma, and malignant subtypes, such as carcinomas, sarcomas and lymphomas	[13]
Healthy dogs (8 cases), dogs with nonmalignant diseases (13 case), and dogs with malignant tumor (20 cases)	Serum samples	Ki-67	Overexpressed in malignant tumors	Ki-67 concentrations increase compared with the healthy group	[18]
Dogs with benign (23 cases) and malignant tumors (43 cases)	Tissue Samples	Ki-67 and PCNA	Upregulated in malignant tumors	proliferation indexes of Ki-67 in malignant tumors: 3–47%; PCNA 21–69%, significant association between Ki-67 and PCNA expression level, and clinicopathological factors	[23]
Dogs with primary mammary carcinomas (29 case), lymph node and metastasis (25 cases)	Tissue samples	Ki-67, EGFR, HER-2, COX-2 and	Ki-67, HER-2, Cox-2 and in paired lymph node metastasis	HER-2, Cox-2 and Ki67 correlated with tumor aggressiveness	[40]
Canine mammary carcinomas (65 cases)	Tissue samples	Ki-67, PCNA, ER, Bcl-2, Caspase 3, 8, 9	Heterogenous expression throughout samples	prognostic significance of Bcl-2 and PCNA expression in CMTs	[41]

Table 1. continued

Dogs with primary mammary tumors (30 cases)	Tumor and adjacent tissue	Bcl-2, PCNA, TP53, ER, cytokeratin – epithelial differentiation marker	Upregulation Cytokeratin – downregulated	Overexpression of Bcl-2 and p53 promote resistance to therapy, loss of differentiation and apoptotic evasion	[42]
Dogs with mammary adenocarcinoma (50 cases adenocarcinoma and 21 cases of soft tissue fibrosarcoma)	Tissue samples	MCM-3	Upregulated in mammary adenocarcinomas	correlation of marker expression and grade of malignancy	[34]
Dogs with mammary malignancies (12 cases, precancerous and cancerous)	Tissue samples	Cyclin D1	Upregulated	Positive correlation between mitotic activity and cyclin D1 expression levels	[31]
Canine Mammary Carcinomas (90 cases)	Tissue samples	HER2	Upregulation	increased number of HER2 gene copies	[39]

Table 2. Relevant examples of biomarkers involve hypoxia, angiogenesis, EMT, invasion, and metastasis, which have implications for CMT management.

Marker	Sample type	Expression profile	Effects	Ref.
VEGF	Serum	Upregulated	Prognostic marker, predict tumor progression	[53]
	Tissue samples	Upregulation	Promote carcinogenesis and tumor progression; promote angiogenesis	[77]
VEGFA, VEGFR2	248 formalin-fixed paraffin-embedded CMT samples	Upregulated	Vasculogenic Mimicry was correlated with increased expression of VEGFA and VEGFR2	[63]

Table 2. continued

VEGFB	mammary gland carcinomas versus adjacent tissue	Upregulated	Correlated with tumor size, grade, and absence of metastasis	[62]
VEGF, Cox-2,	70 tumors (28 benign and 42 malignant)	Upregulated	COX-2 upregulation correlates with VEGF and tumor angiogenesis, promote aggression in malignant tumors.	[65]
Cox-2	Primary and metastatic canine mammary tumors	Upregulated	increased microvessel density increased Cox-2 expression decreased overall survival	[74]
	FFPE	Upregulated	Correlated with angiogenesis; worse prognosis and shorter overall survival	[74]
COX-2 and EGFR	43 malignant CMTs	Upregulated	Associated with high-nuclear grade, high-histological grade and the presence of lymph node metastasis	[66]
EGFR	61 CMT tumors	Upregulated	Association with clinical stage, tumor size, histological and mitotic grade of malignancy	[64]
ZEB1 and ZEB2	M5, M25 and CF41.Mg cell lines	Upregulated	Positive correlation between ZEB1 and ZEB2 and the tumorsphere number and size	[59]
Galectin-3	Primary and metastatic canine mammary tumors	Upregulated	Regulated cell-cell and cell-ECM adhesion; promotion of angiogenesis, cell proliferation and resistance to apoptosis	[31]
TGF-β1	Tissue samples	Upregulated	higher histologic grades decrease of survival rate after 2 years	[78]
IL-35	FFPE	Upregulated	Carcinogenesis and worse prognosis	[79]
IL-6, IL-8	Serum and tissue	Upregulated	Stimulation of tumor cells growth Promotion of loco-regional relapse and metastasis	[71]

Table 3. Relevant examples of altered miRNAs and lncRNAs in CMTs

Marker	Sample type	Marker and expression profile	Effects	Ref.
mir-125a	Tissue samples	Downregulated	Promote tumorigenesis and metastasis	[98]
miR-21	Tissue and serum samples	Upregulated	Prognostic markers for CMT	[93]
	Tissue samples	Upregulated	Promote tumorigenesis	[99]
miR-29b	Tissue samples	Upregulated	Promote tumorigenesis	[99]
miR-19b	Serum sample	Upregulated	Increase overall survival	[92]
miR-124	CHMp and CHMm cell lines	Downregulated	inhibition of cell proliferation and EMT Suppression of the CDH2 expression	[94]
miR-497	CMT1211 and CMT 7364	Downregulated	Induce apoptosis via IRAK2/NF-κB	[85]
lncRNA34977	CHMp and CHMm	Upregulated	Promoter of proliferation, invasion and migration	[89]
LncRNA-42060	CHMp, CHMm, CHMpTAM and CHMmTAM	Upregulated in drug-resistant cells and tumor tissues	Regulated tamoxifen sensitivity miR-204-5p/ SOX4	[97]

CONCLUSIONS

Using molecular markers in CMTs can improve diagnosis, predict treatment response, and inform therapeutic decisions. These markers can provide information about the tumor's genetic makeup, which can help identify the specific cancer subtype, predict its behavior, and determine the most appropriate treatment. Ki-67, EGFR, HER-2, and COX-2 represent the most studied biomarkers in CMTs. However, the identified biomarkers have yet to demonstrate perfection, possibly due to studies conducted on limited subject cohorts, worsened by using different molecular techniques. This underscores the need for additional investigations to comprehend these markers' significance and clinical usefulness in CMTs.

MiRNAs and lncRNAs represent another group of biomarkers with promising results. They can act as oncogenes or tumor suppressor genes with high sensitivity and

specificity, possibly detecting miRNAs in both blood and tissue. The small number of studies investigating cancer stem cells as biomarkers in CMT proves the need to establish a common method for their evaluation because the studies carried out so far indicate conflicting results regarding their level of accuracy as biomarkers.

In summary, novel promising biomarkers can be used in the diagnosis and targeted therapy of CMTs. For this, standardization of clinical studies is needed to provide a more detailed understanding of the tumor's genetic makeup and lead to improved outcomes.

Authors' contributions

LMG, CB, and IBN contributed to the study's design and conception, while EI, OZ, and CB were involved in data curation and visualization. All authors participated in writing and revising the manuscript, which they then read and approved as final.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Statement of Informed Consent

The owner understood procedure and agrees that results related to investigation or treatment of their companion animals, could be published in Scientific Journal Acta Veterinaria-Beograd.

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MOLEKULARNI MARKERI TUMORA MLEČNE ŽLEZDE PASA

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Tumori mlečne žlezda kuja (CMT) su jedna od najčešćih neoplazmi pasa. Nažalost, trenutna dijagnoza se često postavlja u kasnijim fazama i postoji hitna potreba za sveobuhvatnijim podacima o opcijama lečenja kako bi se poboljšala ukupna prognoza. Shodno tome, rano otkrivanje ovih tumora je ključno za poboljšanje ishoda lečenja i stope preživljavanja. Kao takvi, biomarkeri su od suštinskog značaja za poboljšanje dijagnoze, lečenja i prognoze CMT-a, što je razlog zbog kojeg su potrebna dalja istraži-

vanja kako bismo poboljšali naše razumevanje bolesti. Najviše su izučavani biomarkeri za CMT koji se procenjuju iz uzoraka seruma i tkiva koristeći različite molekularne pristupe i odnose se na proliferaciju i ćelijski ciklus. Nekoliko biomarkera je takođe opisano u vezi sa oštećenjem ćelija, autofagijom i apoptotičkim putevima, hipoksijom, angiogenezom, EMT, invazijom, metastazama ili karakteristikama stem ćelija. Sve u svemu, biomarkeri su pokazali potencijal da se koriste kao alat za rano otkrivanje tumora mlečne žlezde pasa. Međutim, potrebna su dalja istraživanja da bi se potvrdili ovi biomarkeri i razvili osetljivi i specifični dijagnostički testovi. U tom smislu, imali smo za cilj da sveobuhvatno pregledamo poznate biomarkere i njihovu ulogu u CMT-u. Takođe smo podstakli dalja istraživanja pouzdanih biomarkera koji bi mogli da poboljšaju ishode lečenja i stope preživljavanja pasa sa ovom bolešću.