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Review Article

Consensus regarding the diagnosis, prognosis and treatment of canine mammary tumors: benign mixed tumors, carcinomas in mixed tumors and carcinosarcomas

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Abstract

Mammary neoplasms are the most frequent tumors in female dogs. Of these neoplasms, benign mixed tumors (BMTs) and carcinomas in mixed tumors (CMTs) represent a large proportion of small animal oncology diagnoses. Together with carcinosarcomas (CSs), these three neoplastic entities are characterized by the proliferation of benign or malignant epithelial, myoepithelial, and mesenchymal cells, depending on their histological types. This histological heterogeneity, in addition to their molecular heterogeneity, confers these tumors with distinct biological behavior, which results in the need for different clinical and therapeutic approaches. The present consensual document elucidates the oncological issues related to the diagnosis, prognosis, and treatment of BMTs, CMTs, and CSs of the canine mammary gland.

Key words: mixed tumours, dogs, mammary gland, veterinary, oncology.

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Introduction

Mixed tumors are the most frequent neoplasms of the female dog mammary gland. These tumors present with a complex histological pattern, comprising epithelial, myoepithelial and mesenchymal elements that might undergo malignant transformation, giving rise primarily to CMTs and less frequently to CSs and sarcomas in mixed tumors (8, 9, 44). Defining the criteria to evaluate the various cellular elements involved in mixed tumors as well as the factors that contribute to malignant transformation is extremely relevant for understanding the behavior and progression of these types of neoplasms. The first Consensus for the Diagnosis, Prognosis, and Treatment of Canine Mammary Tumors (8) discussed the criteria that could be used to guide and standardize diagnoses and treatments. In the second Consensus (10), different oncology groups/services in Brazil presented results concerning the application of the first consensus and the difficulties associated with its application. According to the different groups, mixed tumors (benign mixed tumors BMTs), carcinomas in mixed tumors (CMTs), and carcinosarcomas (CSs) represented the largest proportion of cases and the point of greatest divergence in diagnostic routine (10). How can BMTs be differentiated from CMTs? How can *in situ* carcinomatous areas be differentiated from invasive CMTs? What are the criteria for diagnosing CSs? The correct histological classification of mixed tumors is of extreme importance given that histological variations confer differences in prognosis and treatment. In light of new questions and the search for understanding the prognostic and predictive factors of mixed mammary tumors in female dogs, the Third Mammary Pathology Meeting (III Encontro de Patologia Mamária) was held on 20 and 21 October 2016 in Salvador, Bahia, Brazil. This meeting was organized by the Post-Graduate Program in Animal Science in the Tropics (Federal University of Bahia; UFBA) and the Laboratory of Comparative Pathology (Federal University of Minas Gerais; UFMG) with the support of the Brazilian Association of Veterinary Pathology (ABPV) and the Brazilian Association of Veterinary Oncology (ABROVET). The purpose of this event was to bring together university professors, researchers, private professionals and graduate students working in clinical practice, surgery, and pathology directed towards mammary oncology to discuss the morphological aspects as well as prognostic and predictive factors of mixed tumors associated with the canine mammary gland.

Clinical behavior

Clinical staging

Mammary neoplasm staging in female dogs seeks to evaluate the primary tumor size, the involvement of regional lymph nodes, and the presence of distant metastases. Staging enables disease prognosis and treatment planning. The tumor, lymph node, and metastasis (TNM) system of the World Health Organization for canine mammary tumors, initially proposed by Owen (1980) (50) and adapted by Cassali et al. (2014) (13), is currently used. This system was formulated only for canine epithelial tumors; however, various researchers use this staging criterion for sarcomas, although it is not recommended for this histological entity.

When evidence of regional lymph node metastases exists, a significant drop in survival expectancy occurs compared with dogs that test negative for metastases in lymph nodes or distant sites (39, 59, 66). Recently, staging was related to prognosis in female dogs diagnosed with malignant mammary neoplasms, where those with stages IV or V were associated with a lower overall survival rate than those with stages I, II, or III (49).

Early and complete surgical excision is curative in most cases of canine mammary BMTs and CMTs (43). In the case of BMTs, late excision might enable the malignant transformation of the epithelial component, giving rise to CMTs with worsening disease prognosis. Little is known about the progression of CSs; however, the possibility of a malignant transformation of the mesenchymal component in CMTs is not excluded. Therefore, it is important to identify cases that require adjuvant therapies for all three histological subtypes, in addition to surgical removal; thus, the oncologist should always request a diagnosis, histological grading, and complementary exam (6). For this analysis to be possible and detailed, the oncologic surgeon must process the sample to be sent to the pathologist according to the following criteria: (1) To analyze the mammary nodules, lymph nodes, and surgical margins, the recommendations of (22) that are described in the 2013 Consensus should be followed; and (2) all mammary glands in the chain undergoing surgical excision should be evaluated, even if they do not contain macroscopically detectable tumors.

Morphological criteria and molecular profile

BMTs

BMTs are generally encapsulated and microscopically characterized by the presence of benign epithelial (ductal, acinar, or both types of cells), myoepithelial and mesenchymal elements, with the formation of cartilage, bone, or both, possibly in combination with myxoid fibrous tissue and adipose tissue (Fig. 1). In these tumors, the epithelial component might reveal low cellular atypia and a low mitotic index (45, 46).

The epithelial component might exhibit various growth patterns, and the lobules of the gland are generally poorly preserved. These components are often compressed by the mesenchymal counterpart of the tumor, which

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acquires the appearance of an invasive pseudo-adenocarcinoma. In some cases, apocrine, squamous, or both types of metaplasia are observed.

Proliferating myoepithelial cells can have a fusiform or star-like appearance and are often embedded in an abundant extracellular matrix (myxoid matrix). The proliferation of myoepithelial cells associated with the presence of myxoid matrix is a precursor of the ectopic cartilage observed in BMTs, suggesting that it is the result of the (myo)epithelial-mesenchymal transition (4, 15, 20, 33).

Table 1. Mammary neoplasm staging in female dogs.

STAGE	TUMOUR SIZE (T)	NODAL STATUS (N)	DISTANT METASTASES (M)
1	T1 < 3 cm	N0	M0
2	T2 3-5 cm	N0	M0
3	T3 > 5 cm	N0	M0
4	Any T	N1 (positive)	M0
5	Any T	Any N	M1 (presence of metastases)

Source: Cassali et al. (10)

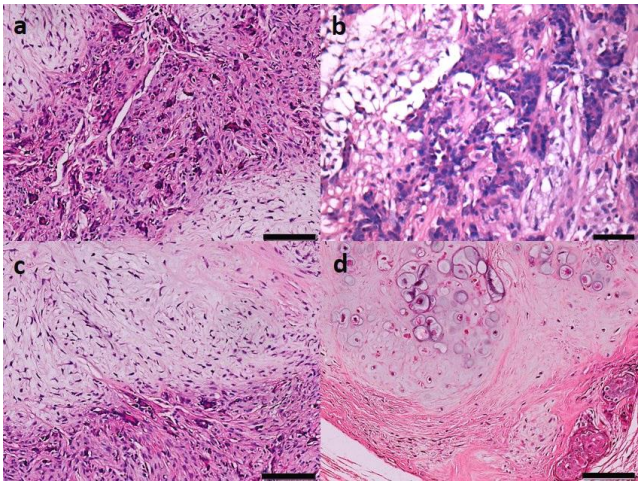

Figure 1. (a) Benign mixed tumors (BMT) in canine mammary gland. HE, bar 100 µm. (b) Benign epithelial cells in benign mixed tumor. HE, bar 50 µm. (c) Benign mixed tumors presenting myoepithelial cells proliferation with mixoid matrix areas. HE, bar 100 µm. (d) Chondroid metaplasia in BTM. HE, bar 100 µm.

Cartilage is present in the form of nodules or plaques of variable sizes, with a discrete-to-moderate population of well-differentiated chondrocytes and chondroblasts. Bone tissue, when observed, is represented by osteoid-producing osteoblasts, well-differentiated trabeculae and mineralized bone. Bone formation occurs via the endochondral ossification of pre-existing cartilage formed by the differentiation of myoepithelial cells or the intramembranous ossification of the connective tissue of the stroma (27). The presence of bone marrow with haematopoietic tissue and intermingled adipose tissue are observed in some cases (2, 28).

CMTs

Previously, CMTs were considered malignant mixed tumors. This denomination was also applied to CSs; therefore, the two were considered synonymous (31, 43, 47). Misdorp et al. (1999) (44) observed that the presence of sarcomatous elements and the higher frequency of metastases justified the separation into two histological types: CMTs (or complex carcinomas) and CSs. In CMTs, only epithelial cells are malignant, which differs from CSs, where both the epithelial and mesenchymal components exhibit malignancy (45) (Fig. 2). The probable common origin of these tumors explains the existing morphological similarities; however, differential diagnoses are difficult.

In the classification proposed by Misdorp et al. (45) and adopted by the Consensus (8, 10), the term "malignant mixed tumor" was discontinued, and the carcinomas associated with BMTs began to be called CMTs. Thus, according to the current classification system, CMTs are characterized by the focal or nodular development of epithelial cells, with different degrees of malignancy associated with a primary BMT (10).

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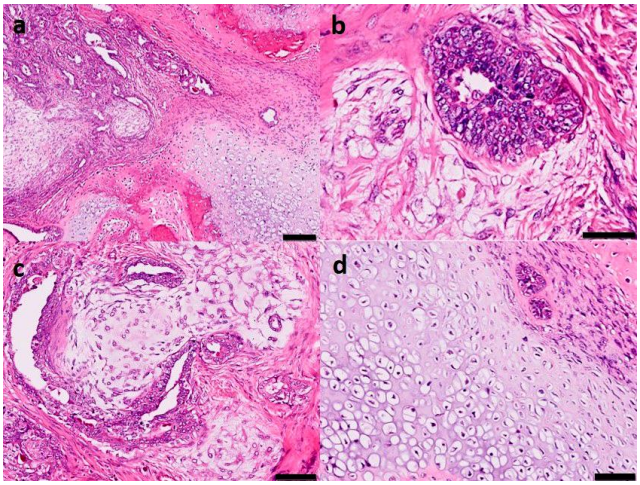

Figure 2. (a) Carcinoma in mixed tumor (CMT) in canine mammary gland. HE, bar 200 µm. (b) *In situ* malignant epithelial cells proliferation in carcinoma in mixed tumor. HE, bar 50 µm. (c) Benign myoepithelial cells proliferation adjacent to carcinomatous cells in carcinoma in mixed tumor. HE, bar 100 µm. (d) Carcinoma in mixed tumor presenting chondroid metaplasia and invasive carcinomatous area in the adjacent stroma. CMT. HE, bar 100 µm.

Microscopically, CMTs are composed of a malignant epithelial component and a benign mesenchymal component that can be represented by cartilage, bone, or adipose tissue in the same way as that observed in BMTs. The epithelial component is characterized by the foci or nodules of cuboidal to columnar epithelial cells with varied pleomorphism, nuclear atypia, and atypical mitoses that arise in BMTs. Carcinomatous proliferation can invade or even completely replace the pre-existing benign lesion at the time of a histopathological examination. Higher cellularity, pleomorphism, mitotic index, foci of necrosis, and an infiltrative growth pattern allow BMTs to be differentiated.

In these tumors, carcinomatous proliferation can exhibit *in situ* or infiltrative growth. *In situ* carcinomatous areas are characterized by a malignant epithelial proliferation in the ductal or lobular units of the mammary gland, occupying the entire lumen without the discontinuity or absence of the basement membrane (10, 58). *In situ* carcinoma areas with microinvasion areas are characterized by a discontinuation of the basement membrane and the layer of myoepithelial cells combined with the presence of a small grouping of epithelial cells that invade the adjacent stroma (<1 mm; 32). The infiltrative pattern is evidenced by the loss of continuity of the myoepithelial and basal layers, combined with the invasion of neoplastic cells in the stroma, which can completely replace the pre-existing benign lesion (8). Thus, an evaluation of the integrity of the myoepithelial cell layer is important in the differential diagnosis between *in situ* and infiltrative lesions.

Malignant transformation of BMTs

The factors that determine the malignant transformation of BMTs have stimulated research (3, 44, 52). However, few studies have examined the malignant progression of these neoplasms in dogs (4). As early as the 1970s, however, Moulton et al. (1970) (47) hypothesized that if mixed tumors had sufficient time to grow, then they could undergo malignancy. Later, Genelhu et al. (2007) (26) and Bertagnolli et al. (2009) (3) observed molecular changes that might contribute to the transformation of BMTs, such as the loss of expression of p63, ΔNp63, E-cadherin, and β-catenin.

One study showed that the overexpression of epidermal growth factor receptor (EGFR) via malignant epithelial cells might be an early event in the carcinogenesis of mixed tumors. Moreover, changes in the expression of this molecule might be crucial in the malignant transformation process within the epithelial component of this histological type (4).

The origin of the malignant transformation process remains unknown; however, the phenotypic evaluation of myoepithelial cells, in addition to extracellular matrix components, has been the subject of studies seeking to clarify the mechanisms involved in the biological behavior of these tumors (14, 15, 16, 20, 48).

The myoepithelial cells in premalignant lesions and *in situ* carcinomas surround the epithelial structures, acting as a barrier that prevents the conversion of *in situ* tumors in invasive carcinomas (29, 42). This suppressive ability of myoepithelial cells, likely depends on their complete differentiation, and changes in their expression pattern might lead to a reversal of their function (i.e., undifferentiated myoepithelial cells might promote tumor progression rather than suppress it; 29).

The essential condition for stromal invasion is the rupture of the basement membrane and the myoepithelial cell layer (41). In human medicine, the evaluation of the integrity of the myoepithelial/basal cell layer is often an important tool in the differential diagnosis between *in situ* and invasive malignant lesions (61, 67). The determination of the invasion foci of mammary tumors enables the prediction of biological behavior. Invasion foci might be associated with metastasis and unfavourable prognosis compared with *in situ* carcinomas (34).

The use of antibodies that identify proteins expressed in myoepithelial cells (e.g., p63, alpha-smooth muscle actin, and low molecular weight cytokeratins) has aided the identification of these areas in humans and canines (3, 24, 26, 52, 65). Bertagnolli et al. (2009) (3) observed a decrease in p63 expression in female dog CMTs, suggesting a loss of myoepithelial cells in these areas and supporting the invasive and progressive character of these tumors. However, the mechanisms that culminate in the descontinuity of this layer are poorly understood (41). Studies of mammary neoplasms of the

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human breast show a decrease in the expression of oestrogen receptors via epithelial cells and tumor suppressor proteins (e.g., maspin, WT-1, and p73) near areas with the loss of myoepithelial cells, which contributes to tumor aggressiveness and invasiveness (41, 42, 65).

CMTs versus other histologic types

Cavalcanti (2006) (11) evaluated CMTs along with all of the histological subtypes of carcinomas (solid, papillary, tubular, micropapillary, and anaplastic) in an analysis of histological type and overall survival. A total of 121 carcinomas histologically classified according to Misdorp et al. (1999) (45) were examined. The most frequent histological type was CMT with 39 cases (32.23%), followed by tubular carcinomas with 32 cases (26.01%), solid carcinomas with 25 cases (20.66%), papillary carcinomas with 20 cases (16.26%), and micropapillary carcinomas with five cases (4.06%). Some tumors in this evaluation had a classification of more than one histological type. Among the 21 cases (17.07%) of composite classification, the tumor received a single classification for survival analysis. In cases of multiple tumors, the histological type with worse prognosis was considered (43). A highly significant relationship was observed between histological type and animal survival ($p<0.00001$).

The increasing order of malignancy and decreasing order of survival time found was CMTs (at 1,800 days; the median was not reached), tubular carcinomas (median reached at 1,380 days), papillary carcinomas (median reached at 820 days), solid carcinomas (median reached at 270 days), and micropapillary carcinomas (median reached at 90 days; Fig. 3).

In fact, that study observed that the animals with CMT presented with a favorable prognosis compared with animals with other carcinomas, and the presence of CMT was considered a protective factor regarding the risk of animal death due to disease. In human histological classifications, CMTs correspond to matrix-producing metaplastic carcinomas, and simple carcinomas correspond to ductal carcinomas. Patients with certain subtypes of metaplastic carcinomas show better prognoses than those with ductal carcinomas (63).

Because CMTs consist of the malignant transformation of the epithelial component in BMTs, this malignancy might give rise to the usually focal carcinomas of the solid, tubular, papillary, and micropapillary subtypes surrounded by the BMT. However, these neoplasms are expected to be part of a lesion with benign behavior and show a better prognosis; furthermore, animals with this type of neoplasia show a longer survival time (11).

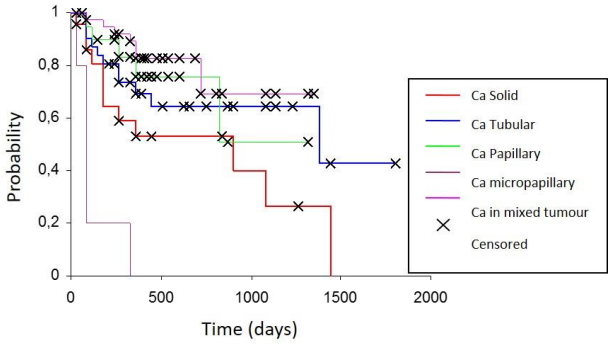

Figure 3. The overall survival curves of animals with mammary carcinomas classified by histological type.

CSs

CSs are uncommon neoplasms in the female dog mammary gland, compared with CMTs, and their clinical and pathological characteristics are similar to those described in humans. They are rare tumors in women and show a worse prognosis than carcinomas (62).

Currently, the denomination “CSs” is used to describe mixed tumors with both malignant epithelial and mesenchymal components (45). These tumors can be macroscopically well circumscribed and non capsulated, with a nodular appearance or infiltrative borders (63). The epithelial component of these tumors can show variable growth and differentiation patterns including adenomatous, solid, squamous, mucinous, and anaplastic, usually revealed as invasive carcinomatous areas as well as *in situ* carcinomatous areas, regardless of infiltrative arrangement (Fig. 4) (46, 63). The sarcomatous component can also vary from fibrosarcomatous to chondrosarcomatous to osteosarcomatous (11, 46). The presence of myxoid matrix may be an indicator that these tumors are derived from CMTs (44). The rate of metastasis is relatively high compared with CMTs (11). The metastasis of the epithelial component spreads via lymphatic vessels to the regional lymph nodes and the lung and that of the mesenchymal component spreads via the haematogenous route primarily to the lungs (27).

In routine practice, a difficulty exists in establishing a CS diagnosis. The malignancy criteria of the epithelial component for invasion and histological grading via the system of Elston and Ellis (1998) (19) are well known and used by many pathologists. However, the high cellularity, particularly the atypia of the mesenchymal component, remain controversial. Often, the heterogeneous and pleomorphic aspect of the mesenchymal cells leads to the diagnosis of malignancy.

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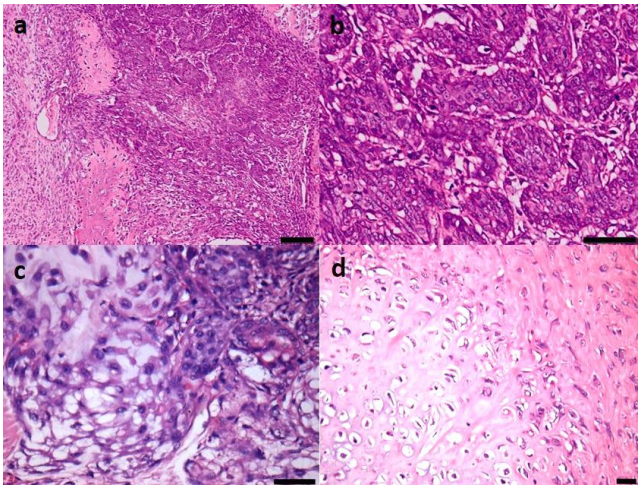

Figure 4. (a) Carcinosarcoma (CS) in canine mammary gland. HE, bar 100 µm. (b) Malignant epithelial cells proliferation presenting pattern solid in carcinosarcoma. HE, bar 50 µm. (c) Pleomorphic myoepithelial cells in carcinosarcoma. HE, bar 50 µm. (d) Carcinosarcoma presenting chondrosarcomatous components. HE, bar 50 µm.

According to Wargotz and Norris, (1989) (63), spindle neoplastic cells should comprise at least 50% of this type of neoplasia for the diagnosis of CSs. Criteria such as high cellularity, atypia, and pronounced cellular pleomorphism, in addition to the high mitotic activity and the presence of necrotic areas, were found in most cases evaluated by Wargotz and Norris and were considered important for the diagnosis of malignancy of the mesenchymal component in CSs of the breast. These authors also indicated that a transition zone between epithelial and malignant mesenchymal components is shown in this histological type.

The degree of differentiation of these tumors is also considered important for the diagnosis of the sarcomatous areas; however, no consensus exists in either the human or veterinary literature regarding the criteria adopted for this purpose. Reports of both women and female dogs have used the criteria of the Nottingham grading system to evaluate only the epithelial component of these biphasic tumors (a term also used for tumors with proliferation of both epithelial and mesenchymal components; 16, 36). Other studies with strictly mesenchymal malignant tumors adopted certain precepts to evaluate the differentiation of sarcomatous proliferation. Based on the cellular pleomorphism, the mitotic index, and the presence of necrosis, Dolka et al. (2013) (17) classified breast sarcomas into two groups: the low malignancy group (well differentiated and moderately differentiated) and the high degree of malignancy group (undifferentiated).

Histopathological grading

Based on the Nottingham system (19), only the malignant epithelial component of carcinomas should be graded. For this purpose, the criteria include tubular formation, nuclear pleomorphism, and mitotic count. Tubular structures should be defined as those that have clear and visible lumen. Score 1 should be attributed to tumors with more than 75% of the carcinomatous area formed by tubules; score 2 represents tumors between 10%-75%; and score 3 denotes those between 0%-10% of the tumor area. To analyze nuclear pleomorphism, the size and shape of the nuclei of normal epithelial cells adjacent to the tumor should be observed and used as a parameter. Score 1 should be assigned to small and regular nuclei and uniform chromatin. Nuclei with increased size and variability should be given a score of 2. A score of 3 denotes the presence of cells with marked pleomorphism, with great variation in nucleus size and shape, and with bizarre and vesicular nuclei with multiple nucleoli. Two observers should perform a mitotic count independently, and the final score should be obtained by calculating the mean score. Mitotic figures should be counted in 10 high-power fields (HPFs), preferably selected at the periphery of the tumor where greater cell proliferation activity is observed (19). The score should be assigned according to the number of mitoses detected: score 1 (0-8 mitoses); score 2 (9-16 mitoses); and score 3 (above 17 mitoses). Pyknotic or hyperchromatic nuclei should not be counted because these cells might be related to necrosis or apoptosis processes. When 10 fields are not found for analysis, the total number of mitoses should be considered based on the number of fields evaluated. To obtain the combined histological grade of the tumor, the score for each factor should be summed, resulting in a total value ranging from 3-9. The tumor grade should be allocated based on the following values: 3-5 points: grade I, low grade; 6-7 points: grade II, intermediate degree; and 8-9 points: grade III, high grade.

Histopathological examination

The histopathological description of these neoplasms should primarily consider the location and extent of neoplasms, type of growth, infiltration in adjacent structures, and the cellular types involved (epithelial, myoepithelial, or mesenchymal). The characterization of proliferating cells is extremely important for the outcome of the anatomopathological diagnosis of BMTs, CMTs, and CSs. For CMTs and CSs, criteria such as malignancy, the proliferation pattern of neoplastic cells, cell characteristics such as cellular (variation in cytoplasmic shape) and nuclear (nucleus variation) pleomorphism, anisocytosis (difference in size among cells), anisocariosis (difference in size of nuclei), and anisonucleosis (difference in size among nucleoli) as well as histological

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grade are crucial for defining the stage of differentiation of malignant neoplasms. To identify the areas of invasion, Schiff's periodic acid staining (PAS) might help (7, 14). The number of mitoses, an evaluation of typical and atypical mitoses, and an evaluation of the invading character, either in the adjacent stroma or blood and lymphatic vessels, are also necessary. The presence of necrosis, ulcerative and hemorrhagic areas, and inflammatory infiltrate (constitution, distribution, and intensity) should be reported. The evaluation of the surgical margins should not be neglected. When fragments of lymph nodes or other organs with neoplastic cells similar to those of the primary neoplasia characterizing metastases exist, these should be described, including which cell type is involved and the major characteristics of these cells (23, 55, 57).

Phenotypic classification into molecular subtypes

The heterogeneity of breast cancer is an important challenge faced by pathologists and oncologists; thus, histopathological classifications and grading should be adopted to predict disease progression and response to treatment, especially in tumors with the same histological subtypes (12). The use of the prognostic and predictive immunomarkers oestrogen receptor (ER), progesterone receptor (PR) and human EGFR 2 (HER2) for phenotypic classification of breast cancer in women is already well defined and established in oncology, providing important predictive and prognostic information for better disease management (30, 64).

In light of the above, molecular classifications with immunohistochemical markers for mammary neoplasms of female dogs have also been studied and evaluated. Gama et al. (2008) (25) studied a panel based on five markers (ER, PR, HER2, CK5, p63, and P-cadherin) and determined four molecular phenotypes: luminal A, luminal B, HER2-overexpressed, and basal-like. They found that the subphenotype luminal A presented with a low histological grade and low proliferation rate, characterizing it as being associated with a positive prognosis. The basal-like subphenotype was characterized by a high grade and high proliferation rate as well as positive expression for cytokeratin 5, p63, and P-cadherin, in addition to a lower disease-free time and overall survival. In contrast, the HER2-enriched and luminal B subtypes were associated with higher survival rates.

Im et al. (2014) (37) used six markers (ER, HER2, CK14, P63, α-SMA, and Vimentin) to define six phenotypic groups for mammary gland cancer in female dogs: luminal A, luminal B, HER2-overexpressed, basal-like, and normal-like. They concluded that the low ER expression and HER2 overexpression in female dog mammary gland cancer were associated with worse prognoses. Basal-like neoplasms presented with a high histological grade and intense invasion in the lymphatic vessels, whereas luminal neoplasms showed a low histological grade and little invasion in the lymphatic vessels. The Laboratory of Molecular Cancer Research (LIMC) located at the Faculty of Medicine of São José do Rio Preto, São Paulo, Brazil, together with the Faculty of Agrarian and Veterinary Sciences (FCAV), São Paulo State University (UNESP), Campus de Jaboticabal, São Paulo, evaluated a prognostic phenotypic classification for mammary gland cancer in female dogs with the immunohistochemical markers ER, PR, and HER2. Using these markers, they characterized the phenotypic profiles into luminal A (ER+, PR+ and HER2-), luminal B (ER+, PR+, and HER2+), HER2-overexpressed (ER-, PR-, and HER2+), and triple negative (ER-, PR-, and HER2-).

The samples were collected from 110 adult female dogs of different breeds and ages affected by breast cancer and cared for at the Obstetrics and Reproduction Clinic of the Governor Laudo Natel Veterinary Hospital of the FCAV/UNESP, Campus de Jaboticabal, São Paulo, Brazil, in partnership with the veterinary clinics of São José do Rio Preto, São Paulo, Brazil. Of these samples, 18 were diagnosed as CMTs and three as CSs. The immunohistochemical technique of the study was performed according to Lopes et al. (2015) (40) in which the immunoreactivity of the anti-ER, anti-PR, and anti-HER2 antibodies was observed using Western blotting. The evaluation of the protein expression obtained by the immunohistochemistry technique was based on the semi-quantitative method. For the oestrogen and progesterone receptors, the scoring index proposed by Allred et al. (1998) (1) was used. For the HER-2/neu marker, the semi-quantitative method described by Koeppen et al. (2001) (38) was used. The phenotypic molecular classification of these two histological subtypes characterized the luminal A (48%), luminal B (28%), and triple negative (24%) subtypes. In this sample, the HER2-overexpressed subtype was not obtained.

Regarding prognosis, the triple negative group presented the worst disease progression, with an overall survival time of 300 days (Fig. 5). It was not possible to determine the survival times of the luminal A and luminal B groups because these dogs remained alive during the 18-month follow-up period, except for one in the luminal B group that died. Fig. 6 shows the death rate among the phenotypes: none in luminal A, one in luminal B, and three in triple negative. Importantly, these deaths were associated with complications of lung metastasis. One dog classified as luminal A presented with pulmonary metastasis. Two dogs classified as luminal B presented with complications of the disease (i.e., recurrence and pulmonary metastasis). Three dogs classified as triple negative had pulmonary metastasis. Regarding the disease stages, stage I was more frequent in the luminal group, and stage V was more frequent in the triple negative group (Fig. 7).

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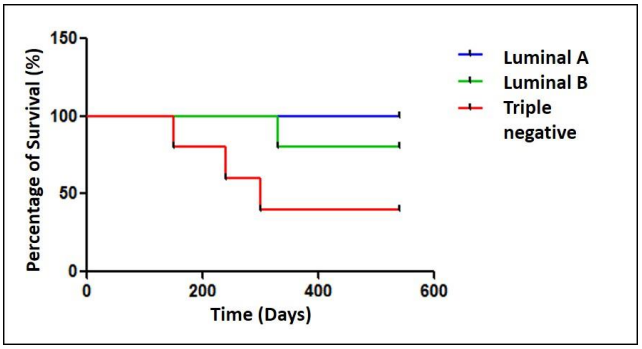

Figure 5. Kaplan-Meier curve to determine luminal A, luminal B, and triple negative phenotype survival rates with regard to the mammary mixed tumor carcinomas and CSs of female dogs.

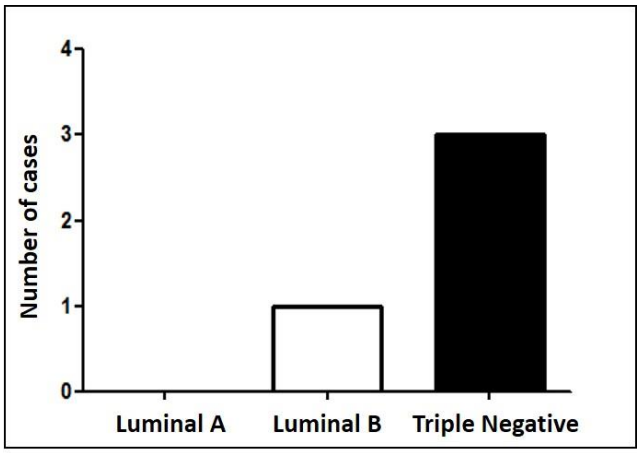

Figure 6. The incidence of death in the molecular phenotypes of CMTs and CSs of female dogs.

Treatment

Regarding the therapeutic approach towards mammary neoplasms, surgery is the treatment of choice for all dogs with mammary tumors, except for those with inflammatory carcinomas (59, 60). The choice of surgical technique depends on the extent of the disease, the size and location of the lesion, and lymphatic drainage (59). Horta et al. (2014) (35) evaluated the influence of surgery in female dogs with mammary tumors and concluded that it does not affect overall survival, disease-free interval, or the development of new lesions. Chemotherapy is indicated for adjuvant treatment in dogs with advanced staging (i.e., regional or distant metastases) or mammary neoplasms with unfavorable prognoses. Certain researchers have suggested chemotherapy for dogs diagnosed with CMT grade II and III (56).

Because of the possibility of metastasis in the lymph nodes that preferentially drain the mammary parenchyma, the removal of these structures is recommended during mastectomy. The lymph node accompanies the inguinal mammary gland when this structure is removed, primarily because of its proximity to the mammary gland. It should be removed in relation to the axillary lymph node, especially when the tumors are located in the thoracic (cranial and caudal) mammary glands and the cranial abdominal mammary gland. A significant obstacle is finding the axillary lymph node, especially when it is not enlarged. To facilitate its localization, the use of 0.1%-2.5% patent blue is recommended at a dosage of 2 mg/kg (13). Complications from dye application are rare, with reports of hypersensitivity reactions occurring in only 0.1%-1.1% of patients. When applied in a large volume or even at the recommended dose, patent blue can temporarily stain the patient's skin, mucous membranes, and urine (5).

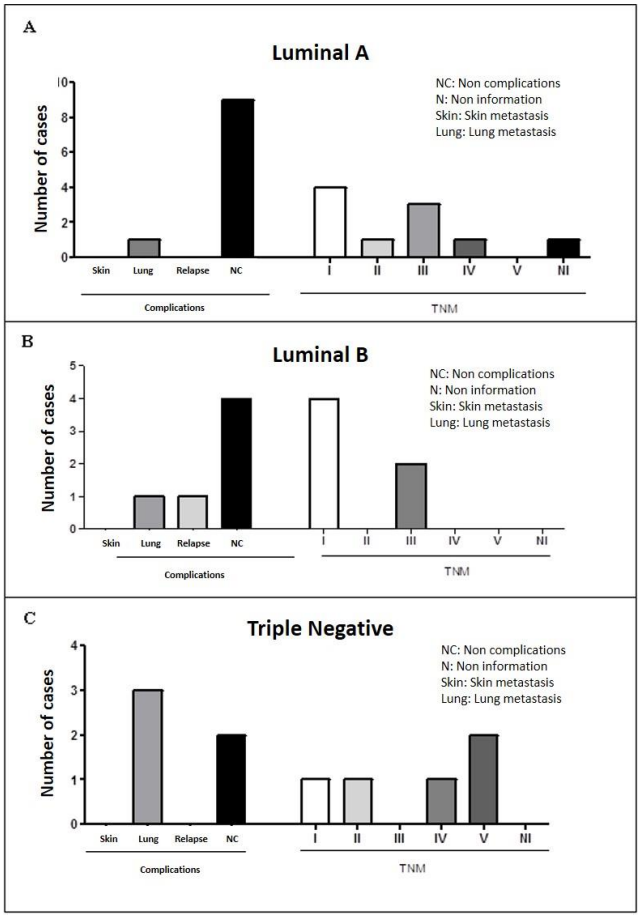

Figure 7. The distribution of the clinicopathological features recurrence (rec.), cutaneous metastasis (cutan.), pulmonary metastasis, and stages (I, II, III, IV and V) in the luminal A (a), luminal B (b), and triple negative (c) phenotypes.

The technique of marking the lymph nodes consists of the inoculation of patent blue subcutaneously in the peritumoral and intradermal regions of the skin covering the neoplasia. For this technique, the neoformation should be virtually divided into four equal

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quadrants (Fig. 8). During the preoperative period, one-fourth of the total volume of the vital marker is inoculated in the superficial intradermal region of each quadrant. Patent blue should be applied between 5 and 10 minutes before the start of the surgical procedure, massaging the area of application for better drainage (Fig. 9).

The "draining" anatomical site is identified by observing the marked lymphatic pathway that corresponds to the location of the lymph node(s). The incision of the area is followed by the separation of the adjacent tissues and the visual identification of the marked lymph nodes. After lymphadenectomy, a second inspection is recommended to check for the presence of other marked lymph nodes (5).

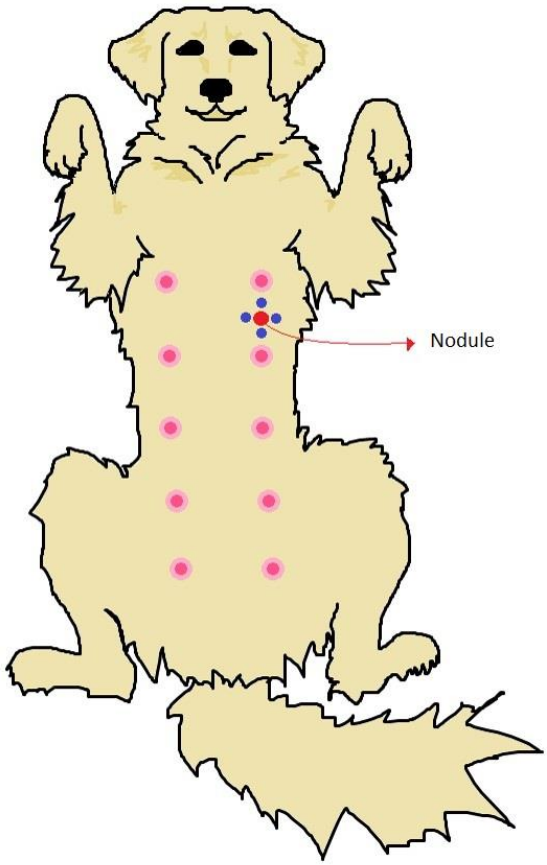

Figure 8. The use of vital dyes is necessary for sentinel lymph node detection: The most suitable vital dye for this purpose is patent blue V because of its low cost, safety, and potential application in dogs. The patent blue technique includes intradermal and peritumoral application at a dose of 2 mg/kg (maximum dose of 1 ml per animal), applied 30 minutes before the beginning of the surgical procedure. The application pattern follows the above scheme: the tumor area is divided into four imaginary quadrants, and 25% of the total dose is applied in the center of each quadrant in the transition region from normal tissue to neoplastic tissue.

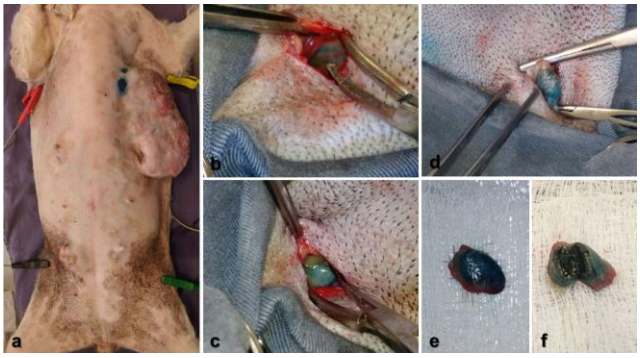

Figure 9. (a) An anaesthetized female dog positioned for surgical mastectomy after the intradermal application of patent blue. (b) The cutaneous incision should be performed in the middle third or caudal middle of the axillary region. After the incision of the dermis, the subcutaneous tissue is separated until the identification of the lymphatic vessels stained with patent blue for use as a guide to identify the axillary lymph node. (c) Using the patent blue stained lymphatic vessels, the axillary lymph node is identified by separating the subcutaneous tissue and pectoral muscle. (d) After the individualization of the axillary lymph node, a mass ligation of the lymph vessels associated with the lymph node is performed. (e) Bluish axillary lymph node due to the use of patent blue dye. (f) Bluish axillary lymph node due to the use of patent blue dye, sectioned in the middle.

According to the 2013 Consensus (10), adjuvant treatment with chemotherapy should be performed in dogs with CMTs with regional, distant, or both types of metastases. Regardless of staging and considering its more aggressive nature, the use of antineoplastic chemotherapy during the postoperative period is recommended for CSs. In female dogs with CMTs presenting with more aggressive histological subtypes (invasive, solid, or tubular micropapillary) or with more extensive invasive areas, the need for adjuvant chemotherapy should be evaluated.

Research from the Federal University of Minas Gerais showed the significant benefit of adjuvant chemotherapy (carboplatin) and antiangiogenic therapy in female dogs with advanced stage CMTs and CSs (Nunes et al., Unpublished data). The combination of antiangiogenic therapies with antineoplastic chemotherapy protocols might benefit the treatment of malignant tumors (51). Treatments with single agents are less effective than multiple chemotherapeutic agents and possible therapies that promote host antitumor defenses (18).

Metronomic chemotherapy protocols are based on the use of the antineoplastic drugs traditionally employed in conventional chemotherapy that are administered orally in low doses at short and regular intervals. The concept of metronomic chemotherapy assumes that antineoplastic drugs alter the tumor microenvironment through antiangiogenic and immunomodulatory effects in addition to the cytotoxic effects they exert on the neoplastic cells.

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The low cost, ease of administration, briefer hospital stay, and, in particular, reduced side effects are important advantages of this therapeutic protocol (53). The authors of this consensus suggest using metronomic chemotherapy for patients with advanced stage metastatic disease and recommend the use of cyclophosphamide (15 mg/m2, po qd) associated with carprofen (4.4 mg/kg, po qd) or firocoxib (5 mg/m2, po qd).

Sterilization

For many years, it was argued that the best way to prevent the development of mammary cancers in female dogs was to perform early sterilization. At present, however, the occurrence of numerous problems (e.g., endocrine, musculoskeletal, and joint changes) is related to spaying female dogs before the first oestrous cycle. Thus, this subject must be better studied to accurately determine the benefits and risks of early sterilization and determine the best time to spay the dog. Although these questions remain unanswered, the authors of this consensus suggest that sterilization before the first oestrous cycle should be avoided and that it be performed between the first and second oestrus when the primary goal is the prevention of mammary neoplasm rather than population control.

Conclusions and perspectives

Mixed tumors are frequent neoplasms of the female dog mammary gland. Despite the high frequency of CMTs in the diagnostic routine, the differences in histological terminology, classification that have emerged over time, and the differentiation of CSs make it difficult to compare data regarding the relapse, malignant transformation, and biology of these tumors. Studies aimed at clinical aspects, malignant transformation, histogenesis, and epithelial- mesenchymal interaction are extremely relevant and must be performed to guide clinical oncology practice and standardize diagnostic and treatment criteria.

The phenotypic classification of the mammary neoplasms of female dogs into molecular subtypes, especially with regard to CMTs and CSs, is important to determine the most accurate disease prognosis. Furthermore, it is expected that personalized therapy will be established in the near future for dogs with mammary gland cancer, as already occurs for women. This advancement will allow clinicians to manage this disease more adequately, thereby improving the survival and quality of life of female dogs.

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