ELAINE DA SILVA SOARES

AVALIAÇÃO DOS FATORES PROGNÓSTICOS DE SOBREVIVÊNCIA DE FÊMEAS CANINAS E FELINAS COM CARCINOMAS MAMÁRIOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae.*

Orientadora: Andréa Pacheco Batista Borges
Coorientadores: Fabrício Luciani Valente
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Andréa Pacheco Batista Borges
Orientadora

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RESUMO

SOARES, Elaine da Silva, D.Sc., Universidade Federal de Viçosa, dezembro 2023. **Avaliação dos fatores prognósticos de sobrevivência de fêmeas caninas e felinas com carcinomas mamários.** Orientadora: Andréa Pacheco Batista Borges. Coorientadores: Fabrício Luciani Valente e Emily Correna Carlo Reis

Apesar do conhecimento já existente acerca dos tumores mamários em cães e gatos, existem poucos estudos que abordem os parâmetros epidemiológicos, clínico-patológicos e histopatológicos associados a exames pré-cirúrgicos de pacientes diagnosticados com essa doença, assim como estudos que associem esses parâmetros com a sobrevida específica do câncer, intervalo livre de doença e sobrevida geral desses pacientes. Diante do exposto, esta tese foi organizada em quatro capítulos em formato de artigo científico. No primeiro capítulo são apresentados os fatores prognósticos para sobrevivência de cães com carcinomas mamários. Nele fatores analisados como a presença de pseudociese, história prévia da doença, estágio clínico avançado (IV–V) e presença de ulceração obtiveram resultados significativos para sobrevida específica-câncer, intervalo livre de doença e sobrevida geral por meio de análise uni-variada e tiveram impacto negativo na sobrevida dos pacientes. A análise multivariada mostrou que a graduação histológica e a idade provaram ser os melhores parâmetros independentes para a avaliação prognóstica de sobrevida específica-câncer e intervalo livre de doença neste estudo. Esses fatores também foram significativos na análise de sobrevida geral. O segundo capítulo aborda a importância do hemograma completo e perfil bioquímico sérico de pacientes felinos com carcinomas mamários como potencial marcadores prognósticos não invasivos e prévios à mastectomia. Nele foram realizadas análises uni-variadas e multivariadas utilizando estes fatores para determinar o efeito de cada parâmetro no tempo de sobrevida de um ano destes animais. Na análise uni-variada, valores dentro da referência de contagem de monócitos, plaquetas e a creatinina foram identificados como fatores prognósticos significativos para sobrevida geral e apenas a creatinina foi significativa para o intervalo livre de doença ($P < 0,05$). Na análise multivariada, as plaquetas e o CHCM (Concentração média de hemoglobina corpuscular) permaneceram como fatores prognósticos independentes para sobrevida geral. Os resultados apresentados sugerem que monócitos, plaquetas e creatinina podem ser importantes marcadores prognósticos pré-cirúrgicos não invasivos, e que a contagem de plaquetas e o CHCM são marcadores prognósticos independentes para carcinoma mamário felino. No terceiro capítulo aborda-se os fatores prognósticos para sobrevivência de gatos com carcinomas mamários. Ulceração, necrose e aderência foram fatores prognósticos negativos e impactantes nesse estudo. A sobrevida geral foi influenciada pela velocidade de crescimento tumoral, pelo tipo de alimentação e pelo histórico de neoplasias mamárias. A graduação histológica não obteve resultado significativo na análise multivariada, mas os carcinomas grau III apresentaram menor intervalo livre de doença. A mastectomia se mostrou curativa em gatas com hiperplasia fibroepitelial e em 5,26% dos carcinomas em estágio inicial, com a mastectomia radical apresentando maior média de sobrevida geral. O quarto capítulo avalia os efeitos da ovariohisterectomia (OVH) concomitante a mastectomia de cães com carcinomas mamários. Nele foram incluídos 102 cães: 44 que foram submetidos à esterilização concomitante à mastectomia (grupo OVH) e 58 que tiveram apenas os tumores removidos, (grupo Não-OVH). As variáveis idade (> 10 anos), grau histológico (3) e grupo não OVH são fatores prognósticos independentes e interferem significativamente no sobrevida específica-câncer, intervalo livre de doença e tempo de sobrevida geral dos pacientes. Assim, a esterilização no momento da mastectomia deve sempre ser considerada em cadelas intactas com tumores mamários, pois tem correlação positiva com maiores sobrevida específica-câncer e intervalo livre de doença na maioria dos casos.

Palavras-chave: Carcinoma; Fatores prognósticos; Tumores mamários; Sobrevida.

ABSTRACT

SOARES, Elaine da Silva, D.Sc., Universidade Federal de Viçosa, December, 2023. **Assessment of prognostic factors for survival of canine and feline females with mammary carcinomas.** Adviser: Andréa Pacheco Batista Borges. Co-advisors: Fabrício Luciani Valente and Emily Correna Carlo Reis

Despite the existing knowledge about mammary tumors in dogs and cats, there are few studies that address the epidemiological, clinicopathological and histopathological parameters associated with pre-surgical examinations of patients diagnosed with this disease, as well as studies that associate these parameters with the cancer-specific survival, disease-free interval and overall survival of these patients. Given the above, this thesis was organized into four chapters in the format of a scientific article. The first chapter presents the prognostic factors for the survival of dogs with mammary carcinomas. In it, analyzed factors such as the presence of pseudocyesis, previous history of the disease, advanced clinical stage (IV–V) and presence of ulceration obtained significant results for cancer-specific survival, disease-free interval and general survival through univariate analysis and had a negative impact on patient survival. Multivariate analysis showed that histological grade and age proved to be the best independent parameters for the prognostic assessment of cancer-specific survival and disease-free interval in this study. These factors were also significant in the overall survival analysis. The second chapter addresses the importance of the complete blood count and serum biochemical profile of feline patients with mammary carcinomas as potential non-invasive prognostic markers prior to mastectomy. Univariate and multivariate analyzes were carried out using these factors to determine the effect of each parameter on the one-year survival time of these animals. In univariate analysis, values within the reference range for monocyte and platelet counts and creatinine were identified as significant prognostic factors for overall survival and only creatinine was significant for the disease-free interval ($P < 0.05$). In multivariate analysis, platelets and MCHC (Mean Corpuscular Hemoglobin Concentration) remained independent prognostic factors for overall survival. The results presented suggest that monocytes, platelets and creatinine may be important non-invasive pre-surgical prognostic markers, and that platelet count and MCHC are independent prognostic markers for feline mammary carcinoma. The third chapter addresses prognostic factors for the survival of cats with mammary carcinomas. Ulceration, necrosis and adhesion were negative and impactful prognostic factors in this study. Overall survival was influenced by the speed of tumor growth, type of diet and history of breast cancer. Histological grading did not yield a significant result in the multivariate analysis, but grade III carcinomas had a shorter disease-free interval. Mastectomy proved to be curative in cats with fibroepithelial hyperplasia and in 5.26% of early-stage carcinomas, with radical mastectomy showing a higher average overall survival rate. The fourth chapter evaluates the effects of ovariohysterectomy (OVH) concomitant with mastectomy in dogs with mammary carcinomas. 102 dogs were included: 44 that underwent sterilization concomitantly with mastectomy (OVH group) and 58 that had only the tumors removed (Non-OVH group). The variables age (> 10 years), histological grade (3) and non-OVH group are independent prognostic factors and significantly interfere with cancer-specific survival, disease-free interval and overall survival time of patients. Therefore, sterilization at the time of mastectomy should always be considered in intact bitches with mammary tumors, as it has a positive correlation with greater cancer-specific survival and disease-free interval in most cases.

Keywords: Carcinoma; Prognostic factors; Mammary tumors; Survival.

SUMÁRIO

1. INTRODUÇÃO GERAL

As taxas de câncer aumentaram em humanos e animais domésticos, tornando esta doença uma das principais causas de morte nessas espécies em todo o mundo (VILHENA et al., 2020). O acesso às vacinas, uma melhor nutrição e cuidados veterinários, bem como um maior interesse dos tutores de animais de estimação permitiram que os cães vivam mais tempo, o que contribui para o aumento de casos de câncer em animais de companhia (HOWARD et al., 2020)., uma vez que a doença está diretamente relacionada à idade. As neoplasias mamárias são um dos tipos de câncer mais prevalentes em humanos, cães e gatos (SAMMARCO et al., 2020). Portanto, elas representam um problema clínico significativo (SORENMO, 2003; BENAVENTE, BIANCHI & ABA, 2016).

O carcinoma mamário é um tipo de tumor maligno da glândula mamária, responsável por mais de 50% dos tumores da glândula mamária canina (SLEECKX et al., 2011, SALAS et al., 2015; BENAVENTE, BIANCHI & ABA, 2016). O carcinoma mamário felino (CMF) é o terceiro tipo de tumor mais comum nessa espécie (MORRIS, 2013; GIMÉNEZ et al., 2010). A neoplasia mamária é um dos tumores mais comuns em cadelas adultas intactas e em cadelas esterilizadas de idade avançada (BULMAN-FLEMING, 2020; PASTOR et al., 2018; SORENMO, 2003) e representa uma das causas mais comuns de morte em gatas de meia-idade e velhas (PETRUCCI et al., 2020a; GIMÉNEZ et al., 2010). A prevalência varia de acordo com a localização geográfica, sendo menor em países onde a ovariohisterectomia é realizada rotineiramente (SORENMO et al., 2019).

No cão é caracterizado por forte invasividade e metástases, especialmente nos linfonodos e pulmões, o que leva a uma maior taxa de mortalidade (MICHISHITA et al., 2023), já o CMF apresenta comportamento agressivo e infiltrativo (HASSAN et al., 2017). A alta incidência de carcinoma mamário canino está intimamente relacionada à idade, hormônios, sexo, hábitos alimentares e histórico reprodutivo (KASZAK et al., 2022). Em cães machos, os tumores mamários são raros, mas geralmente são benignos e apresentam melhores resultados de sobrevivência (KASZAK et al., 2022; PASTOR et al., 2018).

A avaliação prognóstica é geralmente baseada no estágio clínico, tamanho do tumor, estado linfonodal, evidência radiográfica de metástases à distância, linfática ou invasão vascular e exame histopatológico do tumor de acordo com as diretrizes da OMS (Organização Mundial de Saúde) após remoção (SORENMO et al., 2003; QUEIROGA et al., 2011; SANTOS, LOPES & RIBEIRO, 2013). A cirurgia por meio de mastectomia radical continua sendo o tratamento de escolha (GEMIGNANI et al., 2018; PETRUCCI et al., 2020b), visto que a quimioterapia adjuvante não é eficaz na maioria dos casos (MICHISHITA et al., 2016). Por esse motivo, a detecção precoce dos tumores mamários parece ser crucial para o resultado dos pacientes.

Os tumores mamários caninos compartilham diversas características em termos de epidemiologia, patologia, genética tumoral e comportamento biológico comuns com a doença que se desenvolve em humanos (PINHO et al., 2012; NGUYEN et al.; 2017) e expressam as mesmas substâncias, dessa forma, a determinação dos marcadores semelhantes pode ser considerada (PEÑA et al., 2014; NGUYEN et al.; 2017). A determinação de biomarcadores em cães pode ser um marco no diagnóstico precoce de neoplasia, na avaliação da progressão da doença e para modelos relevantes que possam prever a resposta e a potencial resistência a terapias (VARGO-GOGOLA & ROSEN, 2007). É sempre recomendável avaliar mais de um biomarcador, a fim de obter resultados mais confiáveis (KASZAK et al., 2018).

Apesar do conhecimento já existente acerca dos tumores mamários em cães e gatos, existem poucos estudos que abordam os parâmetros epidemiológicos, clinico-patológicos e histopatológicos associados a exames pré-cirúrgicos de pacientes diagnosticados com essa doença, assim como estudos que associem esses parâmetros com a sobrevida específica do câncer, intervalo livre de doença e sobrevida geral desses pacientes, avaliados por meio de análises uni-variadas e multivariadas. Diante do exposto, esta tese foi organizada em quatro capítulos em formato de artigo científico. No primeiro capítulo são apresentados os fatores prognósticos para sobrevivência de cães com carcinomas mamários. O segundo capítulo aborda a importância do hemograma completo e perfil bioquímico sérico de pacientes felinos com carcinomas mamários como potencial marcadores prognósticos não invasivos e prévios à mastectomia. Enquanto, o terceiro capítulo aborda os fatores prognósticos para sobrevivência de gatos com carcinomas mamários. O quarto capítulo avalia os efeitos da ovariohisterectomia concomitante a mastectomia de cães com carcinomas mamários.

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2. CAPÌTULO I:

PROGNOSTIC FACTORS FOR CANCER-SPECIFIC SURVIVAL AND DISEASE-FREE INTERVAL OF DOGS WITH MAMMARY CARCINOMAS

Prognosis of Canine Mammary Tumors

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Abstract

Canine mammary tumors (CMTs) are the most diagnosed neoplasms in dogs; however, there are few studies analyzing the influence of epidemiological, clinicopathological, and histopathological data on cancer-specifc survival (CSS), disease-free interval (DFI) and overall survival (OS) in a large cohort. To contribute to the understanding of the biological behavior of this neoplasm, 385 cases were analyzed, 89% malignant, 4% benign, and 7% non-neoplastic lesions. Among the dogs diagnosed with malignant neoplasms, 86% had early clinical stages (I-III), while 14% had regional or distant metastasis at the time of diagnosis. Carcinoma in a mixed tumor was the most frequent histological type with 44% of the cases and had the best prognosis. Analyzed factors such as the presence of pseudocyesis, previous history of the disease, advanced clinical stage (IV–V), and presence of ulceration obtained significant results for CSS, DFI and OS through univariate analysis and had a negative impact on the survival of the patients. Multivariate analysis showed that histological grading and age proved to be the best independent parameters for the prognostic evaluation of CSS and DFI in this study. These factors were also significant in the overall survival analysis.

Therefore, these parameters should be considered valuable risk and prognostic factors for CMTs.

Keywords: canine, epidemiology, grade, mammary tumors, prognosis

2.1. Introduction

Canine mammary tumors (CMTs) are the most common neoplasms in intact dogs and represent a serious problem in veterinary practice worldwide[1], with malignant tumors responsible for 50% to 70% of CMTs [1–4].

The definitive diagnosis of CMTs is performed through histopathological examination [5, 6]. Late diagnosis makes treatment difficult and reduces the survival of affected animals [7, 8]. The primary treatment to control CMTs is surgery [9-12], which aims to remove the tumor(s) with free margins and prevent the development of new tumors[1]. However, adjuvant therapies can be instituted after surgical treatment [13].

Knowledge of prognostic factors is important to determine therapeutic programs for cancer patients, as it allows the application of different therapeutic modalities in an appropriate and individualized way [14, 15] which can determine the success of the treatment and maintain the quality of life of cured patients [16]. Thus, the objective of the present study was to analyze risk factors and prognostic criteria through univariate and multivariate analyses of epidemiological data, clinicopathological and histopathological characteristics, CSS and DFI of female dogs diagnosed with mammary tumors (MTs), to better understand the behavior of this neoplasm and assist in decision-making.

2.2. Methods

2.2.1. Data collection

Epidemiological and clinicopathological information of 385 female dogs submitted to mastectomy were obtained by consulting the medical records of the Veterinary Hospital of the Federal University of Viçosa (HVT – UFV), Minas Gerais/Brazil, from January 2012 to December 2020. The Ethics Committee for the Use of Animals of the Federal University of Viçosa (CEUA/UFV) approved this study and registered it under protocol number 18/2020.

The clinical data collected were: age at diagnosis, body weight, breed, food type, body condition score (BCS), according to Baldwin et al. [17] on cachectic (1), thin (2), normal (3), fat (4), and obese (5), sterilization status, occurrence of pseudocyesis, hormone administration, and history of previous disease (mammary neoplasm, pyometra or TVT – Transmissible Venereal Tumor). The clinicopathological variables analyzed were: tumor location (thoracic, abdominal, and inguinal mammary glands), number of tumors (single or multiple), tumor size (T1: tumors < 3 cm; T2: tumors between 3–5 cm and; T3: tumors > 5 cm), presence of ulceration, regional lymph node status and presence of distant metastasis.

Clinical staging was performed according to the TNM system (tumor size [T], lymph node involvement [N], and distant metastasis [M]) for CMTs, and categorized into: stage I (T1N0M0, tumors <3 cm), stage II (T2N0M0, tumors between 3–5 cm), stage III (T3N0M0, tumors >5 cm), stage IV (any T N1M0), and stage V (any T, any N0-1M1) [18, 19]. Finally, the animals were classified into early (I–III) or advanced (IV–V) clinical stages.

The staging and search for metastasis in distant organs were performed by chest radiography in the ventrodorsal and right and left laterolateral views, in addition to abdominal ultrasound evaluation, and routine tests such as blood cell count and biochemical profile performed at the time of diagnosis of the mammary neoplasm. The treatment performed on the animals was exclusively surgical resection. The surgical procedures were classified as suggested by Fossum et al. [20], as lumpectomy (removal of only the tumor), regional mastectomy (removal of the affected glands and the ones that shared lymphatic drainage, as well as the lymph node associated with the tumor), or radical mastectomy (unilateral removal of all the mammary chain and associated lymph nodes). The size, location, and number of tumors as well as the clinical status of regional lymph nodes determined the extension of resection.

2.2.2 Histological processing and classification of the samples

The collected samples were fixed in a 10% buffered formalin solution for 48 hours, dehydrated in increasing solutions of ethyl alcohol, cleared in xylene, and embedded in paraffin. Histological sections (3 μm) were obtained and these sections were stained using the hematoxylin/eosin technique.

Tumors were classified according to the consensus for the diagnosis, prognosis, and treatment of CMTs [9, 12, 21]. In addition, the tumors were evaluated according to the Nottingham System classification [22]. The lesion with the worst histological classification and consequent worse prognosis was selected in animals with multiple tumors.

2.2.3. Animal follow-up

After surgery, patients were followed up every six months for a minimum period of 12 months, and the animal's owners were contacted by telephone in cases of loss of clinical follow-up. Local progression was categorized as recurrence close to the previous resection site. DFI was defined as the interval between surgery and the development of recurrences and/or nodal and distant metastasis. CSS was defined as the period between surgical excision of the tumor and disease-related death. OS was defined as the period from surgical removal of the tumor until the patient's death from any cause. Animals that were lost to follow-up were not considered for survival analyses.

2.2.4. Statistical analysis

Comparisons of categorical variables were performed using the chi-square method. Parametric data were submitted for analysis of variance and Tukey's test, while non-parametric data were submitted to Kruskal-Wallis and Dunn's test, considering the significance of $P<0.05$ in Prism 5.0 software (Graph Pad Inc., CA, USA).

For data analysis, the Statistical Analysis System (SAS OnDemand) was used. The overall survival time was analyzed by survival analysis (Lifetest Procedure) and the comparison among the strata was performed by Log–Rank and Wilcoxon tests, the significance level adopted was $P<0.05$.

Explanatory variables were used to predict the probability of survival for at least 1 year by multivariate logistic regression (Logistic Procedure), backward selection was used and only variables that were significant at $P<0.10$ were kept in the final model.

2.3. Results

2.3.1 Epidemiological and clinopathological characteristics of dogs diagnosed with benign and malignant neoplasms

Among the 385 cases analyzed, 7.01% (27/385) corresponded to non-neoplastic lesions, 4.16% (16/385) to benign neoplasms, and 88.83% (342/385) to malignant neoplasms. The median age and weight were 9.55±2.91 years and 11.69±9.97 kg, respectively, with overweight (BCS: 4) and obesity (BCS: 5) observed in 30.73% (110/358) of the cases, with a significant association between the body weight of animals with benign neoplasms and malignant neoplasms (P=0.034). Purebred animals represented 67.04% (240/358) of the cases, with Poodle (90/240), Pinscher (49/240), Yorkshire Terrier (15/240), German Shepherd (12/240), and Dachshund (12/240) being the most affected, while 32.96% (118/358) were considered mixed-breed (Table 1).

About 85.2% (305/358) were intact females, 14.8% (53/358) were spayed, 12.57% (45/358) had a history of pseudocyesis and 17.6% (63/358) received unknown hormonal contraceptive agents. 46.37% (166/358) of the animals received commercial diets, while 31.28% (112/358) were fed a mixture of commercial dog food and homemade food. The occurrence of previous mammary neoplasms was reported in 18.16% (65/358) of the cases (Table 1).

Table 1. Epidemiological information and clinical staging of female dogs diagnosed with mammary gland tumors.

	Benign Neoplasms (n=16)	Malignant Neoplasms (n=342)	P
Age			
≤9.0 years	11(68.75%)	163 (47.66%)	
>9.0 years	5 (31.25%)	174 (50.88%)	0.237
Undefined	0	5 (1.46%)	
Breed			
Mixed-breed	4 (25%)	114 (33.33%)	0.488
Purebred	12 (75%)	228 (66.67%)	
Weight			
≤10 kg	12 (75%)	207 (60.53%)	0.034
>10 kg	4 (25%)	135 (39.47%)	
BCS			
1	0	1 (0.29%)	
2	0	16 (4.69%)	
3	8 (50%)	222 (65.1%)	0.402
4	7 (43.75%)	79 (23.17%)	
5	1 (6.25%)	23 (6.74%)	
Type of food			
Homemade food	0	14 (4.09%)	
Commercial diets	9 (56.25%)	157 (45.91%)	
Commercial diets + meat	1 (6.25%)	5 (1.46%)	0.379
Commercial diets + homemade food	5 (31.25%)	107 (31.29%)	
Commercial diets + snack	1 (6.25%)	59 (17.25%)	
Ovariohysterectomy (OHE)			
No	13 (81.25%)	292 (85.38%)	0.649
Yes	3 (18.75%)	50 (14.62%)	
Pseudocyesis			
Absent	15 (93.75%)	298 (87.13%)	0.435
Present	1 (6.25%)	44 (12.87%)	
Received unknown hormonal contraceptive agents			
No	12 (75%)	283 (82.75%)	0.426
Yes	4 (25%)	59 (17.25%)	
History of previous disease			
Denied	10 (62.5%)	265 (77.49%)	
Mammary neoplasm	4 (25%)	61 (17.84%)	0.075
Pyometra	1 (6.25%)	14 (4.09%)	

TVT	1 (6.25%)	2 (0.58%)	
Surgical technique			
Lumpectomy	1 (6.25%)	21 (6.14%)	0.544
Regional mastectomy	6 (37.5%)	129 (37.72%)	
Radical mastectomy	6 (37.5%)	163 (47.66%)	
Combination of techniques	3 (18.75%)	29 (8.48%)	
Concomitant OHE			
No	11 (68.75%)	234 (68.42%)	0.978
Yes	5 (31.25%)	108 (31.58%)	

BCS, body condition score; TVT**,** transmissible venereal tumor. Similar group characteristics are verified by a nonsignificant P<0.05 using chi square test on categorized variables.

Radical mastectomy was used in 47.21% (169/358) of cases and regional mastectomy in 37.71% (135/358). Concomitant to these procedures, ovariohysterectomy (OHE) was performed in 31.56% (113/358) of cases (Table 1). Single lesions corresponded to 24.02% (86/358) and multiple lesions represented 75.98% (272/358) of the cases, with 66.76% (239/358) located in more than one mammary gland, 15.92% (57/358) in the abdominal and 13.13% (47/358) in the inguinal glands (Table 2).

Tumor size was correlated with the type of neoplasm, as most benign neoplasms (87.5%) and half of the malignant neoplasms (51.75%) were smaller than 3 cm in diameter (P=0.014). Ulceration, distant metastasis, and recurrence were observed, respectively, in 20.47% (70/358), 5.59% (20/358), and 17.88% (64/288) of malignant MTs. Clinicopathological information for the studied neoplasms is detailed in Table 2.

Table 2. Clinicopathological characteristics evaluated in female dogs diagnosed with mammary gland tumors.

	Benign Neoplasms (n=16)	Malignant Neoplasms (n=342)	**P**
Ulceration			
Absent	16 (100%)	272 (79.53%)	0.044
Present	0	70 (20.47%)	
Tumor size			
T1	14 (87.5%)	177 (51.57%)	0.014
T2	2 (12.5%)	69 (20.18%)	
T3	0	96 (28.07%)	
Number of tumors			
Single	2 (12.5%)	84 (24.56%)	0.270
Multiple	14 (87.5%)	258 (75.44%)	
Location of the tumor			
Thoracic	0	15 (4.39%)	0.338
Abdominal	1 (6.25%)	56 (16.37%)	
Inguinal	1 (6.25%)	46 (13.45%)	
Multicenter	14 (87.5%)	225 (65.79%)	
Distant metastasis			
No	15 (93.75%)	323 (94.44%)	0.906
Yes	1 (6.25%)	19 (5.56%)	
Local recurrence			

No	9 (90%)	215 (77.34%)	0.154
Yes	1 (10%)	63(22.66%)	

T1, tumors < 3 cm; *T2,* tumors between 3 – 5 cm; *T3,* tumors > 5 cm. Similar group characteristics are verified by a nonsignificant P<0.05 using chi square test on categorized variables.

Among the non-neoplastic epithelial lesions, 51.85% (14/27) corresponded to lobular hyperplasia and 37.04% (10/27) to adenosis. Benign mixed tumors corresponded to 75% (12/16) of benign neoplasms and among malignant neoplasms,

carcinomas in a mixed tumor (169/342, 49.42%), tubular carcinomas (56/342, 16.37%), papillary carcinomas (34/342, 9.94%), and solid carcinomas (25/342, 7.31%) were the most frequent histological types. Among carcinomas of special types, micropapillary carcinomas represented 17/25 cases. Myoepithelial neoplasms represented 3.51% (12/342) of the cases, and carcinosarcoma was diagnosed in 2.92% (10/342) of the cases, being the most frequent among sarcomas (Supplementary Table 1).

2.3.2. Univariate survival analysis

2.3.2.1. Cancer-specifc survival (CSS) and disease-free interval (DFI)

Animals with non-neoplastic lesions (n = 27) and benign neoplasms (n = 16) were excluded from survival analyses. The loss of clinical follow-up and contact with the animal owners occurred in 18.71% (64/342) of the dogs with malignant MTs. Among dogs with malignant MTs, the following were found after complete follow-up: (1) 27.34% (76/278) were alive without recurrence or metastasis, (2) 8.63% (24/278) were alive but with disease progression, (3) 26.98% (75/278) died due to mammary cancer, and (4) 37.05% (103/278) died of unrelated or unknown causes. As a result, 99 dogs (alive with recurrence and dead from the specific disease) were included in the CSS and DFI analyses,

and for OS, 202 dogs were included.

A significant association was observed between age, CSS and DFI. Younger animals (≤9.0 years) had higher medians of CSS and DFI (1.460 days, confidence interval 95% - IC 95% 730–1.825) and the older (>9.0 years) had the lowest (365 days, IC 95% 365–730, *P* = 0.001; *P* = 0.001, respectively, Supplementary Figure 1A and 1B; Supplementary Table 2). CSS and DFI did not significantly differ according to previous exposure to unknown hormonal contraceptives. CSS probabilities were not significantly different in animals with or without a history of pseudocyesis, however, animals with a history of pseudocyesis showed an increased DFI in comparison to those never experiencing pseudocyesis (*P* = 0.034, IC 95% 60–, Supplementary Figure 1C; Supplementary Table 2).

History of previous disease significantly influenced CSS, but not DFI. Dogs with a history of mammary neoplasm had a lower median of CSS (547 days, IC 95% 210–730) and animals without a history of previous disease had a higher median of CSS (730 days, IC 95% 365 –1.095, *P* = 0.021, Supplementary Figure 1D; Supplementary Table 2).

The type of food consumed by dogs with malignant carcinomas, tumor size, the presence or absence of distant metastases at time of the diagnosis and the performance of OHE before or at the time of mastectomy were not significant for CSS and DFI.

Dogs in the initial clinical stage (I–III) had CSS and DFI (median of 730 days, IC 95% 730–1.095, *P* = 0.006; *P* = 0.013, Supplementary Figure 1E and 1F; Supplementary Table 2) significantly higher than those diagnosed at an advanced clinical stage (IV–V) (median of 365 days, IC 95% 90–730). Dogs diagnosed with carcinoma in a mixed tumor had higher medians of CSS and DFI (1033 and 1095 days) than dogs with other types of malignant MTs.

The histological grading performed in 324 cases of malignant neoplasms resulted in: 36.73% (119/324) classified as grade I, 45.06% (146/324) as grade II, and 18.21% (59/324) as grade III, who had the lowest median of CSS and DFI (365 days, IC 95% 90–730; and 180 days, IC 95% 60–365, respectively). Histological grades I and II had significantly the highest medians of CSS and DFI (*P* = 0.001; *P* = 0.002; Supplementary Figures 1G and 1H; Supplementary Table 2). Animals with ulcerated tumors at the time of diagnosis of the disease had an median CSS and DFI of 365 days (IC 95% 150–365), while in the absence of ulceration the median CSS and DFI of 730 days (IC 95% 730–1.095) (*P* = 0.003; P = 0.001; Supplementary Figures 1I and 1J; Supplementary Table 2). The type of procedure (radical or conservative surgery) performed did not demonstrate a significant prognostic value for CSS and DFI. CSS and DFI information for the malignant neoplasms studied are detailed in Table 3.

Table 3. One-year corrected survival rates in 95 cases (4 failures) with available follow-up data[a].

		Cancer-specifc survival (*n*=95)			**Disease-free interval** (*n*=95)		
		n	Median survival (days)	Median 1-year survival rate *n* (%)	*n*	Median survival (days)	Median 1-year survival rate *n* (%)
Age	≤9.0 years	30	1.460	23 (76.67%)	30	1.460	23 (76.67%)
	>9.0 years	65	365	31 (47.69%)	65	365	31 (47.69%)
	P		**0.001**			**0.001**	
Breed	Mixed-breed	27	730	15 (55.56%)	27	365	13 (48.15%)
	Purebred	68	730	39 (57.35%)	68	730	35 (66.18%)
	P		0.453			0.234	
Type of food	Homemade food	2	380	0	2	197	0
	Commercial diets	43	730	24 (55.81%)	43	730	23 (53.49%)
	Commercial diets + meat	2	2.007	1 (50%)	2	2.007	1 (50%)
	Commercial diets + homemade food	35	365	17 (48.57%)	35	365	14 (40%)
	Commercial diets + snack	13	1.095	10 (76.92%)	13	1.095	9 (69.23%)
	P		0.117			0.082	
Ovariohysterectomy (OHE)	No	78	730	47 (60.26%)	78	730	42 (53.85%)
	Yes	17	365	7 (41.17%)	17	365	6 (35.29%)
	P		0.090			0.078	
Pseudocyesis	Absent	88	730	50 (56.82%)	88	365	43 (48.86%)
	Present	7	1.825	4 (57.14%)	7	1.825	4 (57.14%)
	P		0.182			**0.034**	
Hormone administration	No	81	730	45 (55.56%)	81	730	40 (49.38%)
	Yes	14	730	9 (64.29%)	14	730	8 (57.14%)
	P		0.714			0.636	
History of previous disease	Negative	71	730	42 (59.15%)	71	730	37 (52.11%)
	Positive	24	547	12 (50%)	24	365	11 (45.83%)
	P		**0.021**			0.230	
Clinical Stage (TNM)	Initial (I–III)	74	730	45 (60.81%)	74	730	40 (54.05%)
	Advanced (IV–V)	21	365	9 (42.86%)	21	365	8 (38.1%)
	P		**0.006**			**0.013**	
Surgical technique	Radical mastectomy	48	730	27 (56.25%)	48	730	25 (52.08%)
	Other techniques	47	730	27 (57.45%)	47	365	23 (48.94%)
	P		0.782			0.937	
Tumor size	T1 (<2cm)	49	730	27 (55.1%)	49	730	24 (48.98%)
	T2 (2 – 3cm)	20	547	10 (50%)	20	365	9 (45%)
	T3 (>3cm)	26	730	17 (65.38%)	26	730	15 (57.69%)
	P		0.668			0.698	
Histological type (*n*=72)	Carcinoma in a mixed tumor	42	1.033	31 (73.81%)	42	1.095	27 (64.29%)
	Solid carcinoma	15	365	6 (40%)	15	365	6 (40%)
	Tubular carcinoma	11	730	6 (54.55%)	11	365	5 (45.45%)
	P		0.298			0.264	
Histological grade (*n*=90)	Grade I	32	1.095	22 (68.75%)	32	1.095	21 (65.63%)
	Grade II	27	1.095	19 (70.37%)	27	1.095	16 (59.62%)
	Grade III	31	365	11 (35.48%)	31	180	9 (29.03%)
	P		**0.001**			**0.002**	
Distant metastasis	No	84	730	48 (58.54%)	84	730	42 (50%)
	Yes	11	730	6 (54.55%)	11	730	6 (54.55%)
	P		0.133			0.195	
Ulceration	Absent	67	730	43 (64.18%)	67	730	40 (59.7%)
	Present	28	365	11 (39.29%)	28	365	8 (28.57%)
	P		**0.003**			**0.001**	

[a]Survival values are percentages for cumulative survival determined by Log-Rank test. Significance level adopted was P<0.05.

2.3.2.2. Overall survival (OS)

A significant association was observed between age and OS. Younger animals (≤9.0 years) had higher median of OS (1.095 days, IC 95% 730–1.825) and the older (>9.0 years) had the lowest (365 days, IC 95% 180–730, $P \leq 0.001$, Supplementary Figure 2A). Animals with a history of pseudocyesis had a higher OS compared to those that never had pseudocyesis ($P = 0.039$, IC 95% 365–1.825, Supplementary Figure 2B).

Dogs in the early clinical stage (I–III) had a significantly longer OS (median: 730 days, IC 95% 730–1.095) than those enrolled in an advanced clinical stage (IV–V) (median: 365 days; IC 95% 180 –730, Supplementary Figure 2C). Histological grades I and II had significantly the highest median OS and grade III had the lowest median OS (365 days; IC 95% 210–730) (P=<0.001; Supplementary Figure 2D). Animals with ulcerated tumors at the time of disease diagnosis had a median OS of 730 days (IC 95% 365–730), while in the absence of ulceration, the median OS was 730 days (IC 95% 730–1.095) ($P = 0.002$; Supplementary Figure 2E).

OS was not influenced by hormone administration, tumor size, ovariohysterectomy, histological classification of tumors and distant metastasis. OS information for the malignant neoplasms studied are detailed in Table 4.

Table 4. One-year corrected survival rates in 202 cases with available follow-up data[a].

		Overall survival (*n*=202)		
		n	Median survival (days)	Median 1-year survival rate *n* (%)
Age	≤9.0 years	30	1.095	16 (53.33%)
	>9.0 years	65	365	18 (27.69%)
	P		**<0.001**	
Ovariohysterectomy (OHE)	No	173	730	68 (39.31%)
	Yes	29	730	11 (37.93%)
	P		0.355	
Pseudocyesis	Absent	184	730	70 (38.04%)
	Present	18	1.095	8 (44.44%)
	P		**0.039**	
Hormone administration	No	170	730	70 (41.18%)
	Yes	32	730	9 (28.13%)
	P		0.865	
Clinical Stage (TNM)	Initial (I–III)	170	730	72 (42.35%)
	Advanced (IV–V)	32	365	15 (%)
	P		**0.031**	
Tumor size	T1 (<2cm)	94	730	35 (37.23%)
	T2 (2 – 3cm)	45	730	18 (40%)
	T3 (>3cm)	63	730	26 (41.27%)
	P		0.749	

Histological type (*n*=145)	Carcinoma in a mixed tumor	90	730	40 (44.44%)
	Solid carcinoma	19	365	8 (42.11%)
	Tubular carcinoma	36	730	12 (33.33%)
	P		0.205	
Histological grade (*n*=186)	Grade I	66	1.095	16 (24.24%)
	Grade II	77	730	35 (45.45%)
	Grade III	43	365	20 (46.51%)
	P		**<0.001**	
Distant metastasis	No	186	730	75 (40.32%)
	Yes	16	547	4 (25%)
	P		0.143	
Ulceration	Absent	153	730	65 (42.48%)
	Present	49	730	14 (28.57%)
	P		**0.002**	

[a]Survival values are percentages for cumulative survival determined by Log-Rank test. Significance level adopted was P<0.05.

2.3.3. Multivariate survival analysis

The multivariate Cox regression model included the explanatory variables BCS (score 1–5), histological grade (score I–III), TNM–based clinical stage (initial or advanced), tumor size (score 1–3), pseudocyesis (present or absent), OHE (yes or no), distant metastasis (yes or no), ulceration (present or absent), hormone administration (yes or no), age (years), weight (kg), and number of mammary lesions (n). The model was able to explain significantly the variability seen in the population (P < 0.10). Only the histological grade and age variables remained as independent prognostic factors in the final model for CSS and DFI (Table 5; Figure 1).

Table 5. Analysis of maximum likelihood estimates of multivariate logistic regression to predict the probability of overall survival and disease-free interval for at least 1 year after surgery in dogs affected by mammary tumor.

Cancer-specifc survival			**Disease-free interval**		
Parameter	Estimate	Standard Error	Parameter	Estimate	Standard Error
Intercept	3.6329	1.0338	Intercept	3.8524	1.0272
Grade (1)	0.9109	0.4777	Grade (1)	0.4808	0.3946
Grade (2)	0.2941	0.4453	Grade (2)	0.5794	0.4269
Grade (3)	–	–	Grade (3)	–	–
Age	–0.2307	0.0947	Age	-0.2811	0.0956
c (Area under ROC curve) = 0.816.			c (Area under ROC curve) = 0.823.		

In CSS, the odds ratio for age was 0.794. Each increase in age by 1 year multiplies the risk of event occurrence (cancer-related death) by 0.794, and thus, each decrease in patient age by 1 year enhances the cancer-specific survival probabilities by 1.259 times. The odds ratio for the histological grade is given Table 6.

In DFI, the odds ratio for age was 0.755. Each increase in age by 1 year multiplies the risk of event occurrence (cancer-related death) by 0.755, and thus each decrease in patient age by 1 year enhances the disease-free interval probabillities by 1.325 times. The odds ratio for histological grade is given in Table 6.

Table 6. Odds ratio for Histological grade.

Cancer-specifc survival				Disease-free interval			
Histological grade	1	2	3	Histological grade	1	2	3
1	–	1.853	8.297	1	–	0.906	4.669
2		–	4.478	2		–	5.153
3			–	3			–

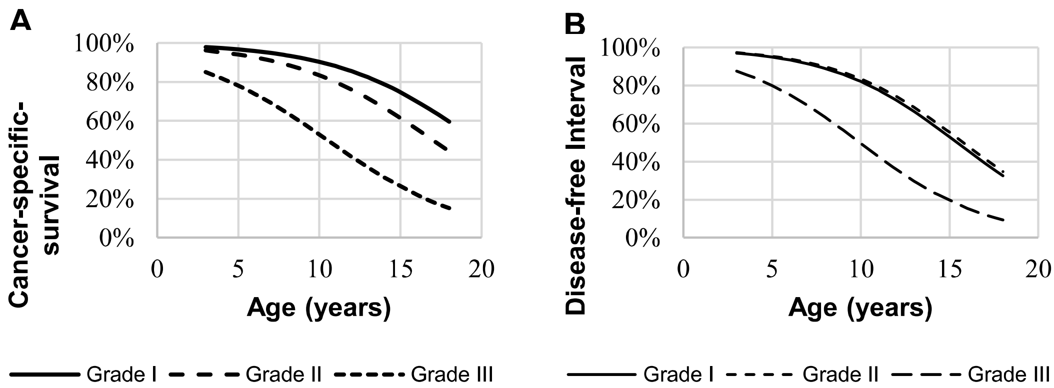

Figure 1. A – Probability of CSS for at least 1 year in dogs affected by mammary tumor according to age and histological grade. **B –** Probability of DFI for at least 1 year in dogs affected by mammary tumor according to Age and Histological grade.

2.4. Discussion

Epidemiological studies and survival with multivariate analysis are scarce, but important to elucidate the biological behavior of CMTs. They might be performed using veterinary records that allow the analysis of significant amounts of data, defining the prognostic and predictive factors, the characterization of neoplasms and the observation of tumor progression, which are important for the definition of CSS and DFI and more appropriate therapy. Thus, the present study is of particular interest due to the cutoff of CMTs felt so far, making it a rare report on disease-free interval, specific survival, and overall survival of CMTs, as most previous studies have focused on the disease-free survival and overall survival only [23–30].

Age is considered a determining factor for the occurrence of MTs, more frequent between 9 and 11 years old [2, 19, 31–33], with a higher occurrence of malignant neoplasms in older animals [15] and benign neoplasms in young animals [4, 32]. In this study, a behavior similar to that described was observed.

Breed is also considered a risk factor for this disease as a result of the existence of a genetic predisposition [2, 34, 35]. In spite of this, in our study, we did not observe differences in CSS, DFI and OS between mixed-breed and purebred animals with MTs, but as expected purebred animals were more frequent [1, 4, 6].

The majority of dogs in this study were not spayed, which could be a risk factor for CSS, DFI and OS since OHE in early life significantly reduces the risk of developing the CMTs [2, 34, 36, 37]. However, the performance or not of OHE in animals with MTs was not significant for CSS, DFI and OS, as observed by Yamagami et al. [23] and Kristiansen et al. [38. In addition, it was observed that the use of unknown hormonal contraceptive agents did not significantly influence CSS, DFI and OS, different from what was expected, since the prolonged use of contraceptives stimulates the synthesis of growth hormone in the mammary gland [39].

Pseudocyesis is implicated in the pathogenesis of CMTs [40–42] and, in this study, proved to be a prognostic factor for DFI and OS. It is still not proven that dogs with a history of malignant MTs. are at greater risk of developing new mammary neoplasms [43, 44]. However, it was observed that the history of MTs. proved to be a prognostic factor for CSS.

Tumors occur more frequently in the caudal abdominal and inguinal mammary glands [45–47], probably because in these glands there is a greater amount of mammary parenchyma and a greater proliferative response to the action of hormones [48], which corroborates the results of this study.

Tumor size is considered an independent prognostic factor, and T1 tumors are associated with a better prognosis [12, 15, 47]. T1 and T2 lesions occurred mainly in carcinoma in a mixed tumor and tubular carcinomas. Animals with nodal and distant metastasis (advanced clinical stage [IV–V]) had lower CSS compared to the initial stage (I–III) of the disease, in agreement with the findings of Nguyen et al. [15]. Animals with an advanced clinical stage (IV–V) should undergo combined therapy with chemotherapy adjuvant to surgery in an attempt to increase OS [12] and CSS.

In the present study, carcinoma in a mixed tumor was the most frequent histological type, corroborating the findings of other authors [8, 39, 49] and presenting a better prognosis and greater CSS, DFI and OS. The histological type of the tumor must be taken into account, since histological variations confer differences in prognosis and treatment [21].

Most MTs. in this study were associated with a low histological grade (I–II), resulting in better CSS, DFI and OS. Tumors with the histological grade III showed a significant reduction in CSS, DFI and OS, as observed by Peña et al. [29] and Nunes et al. [47] in dogs with undifferentiated carcinomas (grade III) that had a worse prognosis than dogs with grade I and II carcinomas. Supporting this data, the application of multivariate logistic regression selected histological grading as an important parameter to assess the clinical outcome. Histological grade was an independent and highly significant prognostic parameter for CSS, DFI and OS.

Ulceration indicates a worse prognosis [9]. Ulceration can be caused by invasive tumor growth or trauma, ischemia, or skin infection, which are characteristics not necessarily associated with aggressive biological behavior [50]. In this study, the presence of tumor ulceration reduced the CSS of the affected animals by 50% and was mainly observed in patients with tumor size 3 and advanced clinical stage (IV–V). Thus, this clinical pathological feature can also be proposed as a prognostic factor for CMTs.

Surgery is the main treatment for MTs [12], except for those with inflammatory carcinomas [19, 51, 52]. CMTs are associated with high rates of morbidity and mortality [9, 15, 53], in this study, a mortality rate of 21.63% was observed one year after the diagnosis of the mammary tumor.

We encountered multiple limitations throughout the process of making this research. For this retrospective study to have a larger number of cases analyzed and, therefore, more effective results, we looked over cases from the years 2012 to 2021, checking the hospital's records and manually selecting all of the female dogs that had a mastectomy. However, during this period, the Veterinary Hospital went through several renovations and changed the system, resulting in the loss of a big percentage of the information available from the previous years.

After that, we had to contact the animal owners of the animals we selected, as there was no follow-up described in the medical records. However, some telephone numbers were out of date or had not been provided and some guardians refused to participate in the study, so 60 animals were excluded due to failure to follow up.

Finally, we had all the information we wanted and selected the histological slides of each tumor to have a more recent and adequate histopathological classification, according to the consensus and literature suggested in the methodology [12]. Many slides disappeared and had to be redone after finding the parafinn blocks, but some materials were lost due to age or poor processing, excluding these animals from the study. After that, we had a total of 385 dogs with all the necessary factors to enter this retrospective study.

(e only course of treatment used in these animals was the surgical removal of their respective tumors (radical or regional mastectomy or lumpectomy). The diagnostic method used consisted only of histological classification. Immunohistochemistry and other techniques are not widely used in our region due to the high cost and difficulty in acquiring antibodies. We recognize that the evaluation of biomarkers is extremely important to predict the biological behavior of cancer and to have a better prognosis for each case, but unfortunately, it is not possible to have it in the daily routine of the UFV Veterinary Hospital, which aims to make veterinary medicine accessible to the local population in need while teaching new professionals.

2.5. Conclusion

Advanced age and histological grading are important factors for the disease, both directly influencing the CSS, DFI and OS of dogs with malignant neoplasm. Older animals had drastically reduced CSS and DFI compared to younger animals, as well as animals with grade I and II tumors had longer survival intervals than those with grade III. Female dogs with a history of previous mammary neoplasm had a lower median of CSS. Clinical staging significantly influenced survival, with dogs diagnosed in the early clinical stage having considerably higher CSS, DFI and OS than those with advanced stage (IV–V). Furthermore, animals with ulcerated tumors had lower CSS, DFI and OS compared to those with an absence of ulceration.

Data Availability

The data used to support the 4ndings of this study are available on request from the corresponding author.

Ethical Approval

All procedures were performed according to the Ethics Committee for the Use of Animals (CEUA), protocol 18/2020.

Conflicts of Interest

The authors declared that they have no conflicts of interest.

Author's Contributions

ESS, CCR, and CERP conducted laboratory analyses and data interpretation; ESS wrote the article; ESS, CCR, and TBS drafted and revised the article draft; CCR and FLDO translated the paper; APBB, SLMC, and FLV revised the manuscript draft and supervised the laboratory work. ESS and APBB planned and designed the study. All authors read and approved the final version of the article.

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Supplementary Materials

Supplementary Figure 1: cancer-specific survival and disease-free interval curve of Kaplan–Meier of female dogs with malignant mammary neoplasms. Malignant mammary neoplasms classified according to: (A) CSS age: ≤9 years (median 1.460 days) and >9 years (median 365 days). (B) DFI age: ≤9 years (median 1.460 days) and >9 years (median 365 days). (C) DFI pseudocyesis: present (median 1.825 days) and absent (median 730 days). (D) CSS disease history: negative (median 730 days) and positive (median 547 days). (E) CSS clinical staging: initial (median 730 days) and advanced (365 days). (F) DFI clinical staging: initial (median 730 days) and advanced (365 days). (G) CSS histological grade: I (median 1.095 days), II (1.095 days) and III (365 days), respectively. (H) DFI histological grade: I (median 1.095 days), II (1.095 days) and III (180 days), respectively. (I) CSS ulceration: present (median 365 days) and absent (median 730 days). (J) DFI ulceration: present (median 365days) and absent (median 730 days). Supplementary Figure 2: overall survival curve of Kaplan–Meier of female dogs with malignant mammary neoplasms. Malignant mammary neoplasms classified according to: (A) age: ≤9 years (median 1.095 days) and >9 years (median 365 days). (B) pseudocyesis: present (median 1.095 days) and absent (median 730 days). (C) clinical staging: initial (median 730 days) and advanced (365 days). (D) histological grade: I (median 1.095 days), II (730 days) and III (365 days), respectively. (E) ulceration: present (median 730 days) and absent (median 730 days). Supplementary Table 1: **h**istopathological classification and frequency in % of 385 cases of mammary tumors diagnosed in female dogs treated at the UFV Veterinary Hospital, which were classified into: Non-neoplastic lesions (*n* = 27/7.01%), benign neoplasms (*n* = 16 /4.16%) and malignant neoplasms (*n* = 342/88.83%). Supplementary Table 2: estimates of life survival and risk functions in 95 cases with available follow-up data: age ≤9.0 years (n = 30) and, age >9.0 years (*n* = 65); history of previous disease: negative (*n* = 71) and, positive (*n* = 24); clinical stage (TNM): Initial (I–III) (*n* = 74) and advanced (IV–V) (*n* = 21); histological grade (*n* = 90): grade I (*n* = 32), grade II (*n* = 27) and grade III (*n* = 31) and; ulceration: absent (*n* = 67) and present (*n* = 28) for cancer-specific survival. Estimates of overall lifetime survival and risk functions in 95 cases with available follow-up data: age ≤9.0 years (*n* = 30) and, age >9.0 years (*n* = 65); pseudocyesis: absent (*n* = 88) and present (*n* = 7); clinical stage (TNM): initial (I–III) (*n* = 74) and advanced (IV–V) (*n* = 21); histological grade (n = 90): grade I (n = 32), grade II (*n* = 27) and grade III (*n* = 31) and; ulceration: absent (*n* = 67) and present (*n* = 28) for disease-free interval. (*Supplementary Materials*).

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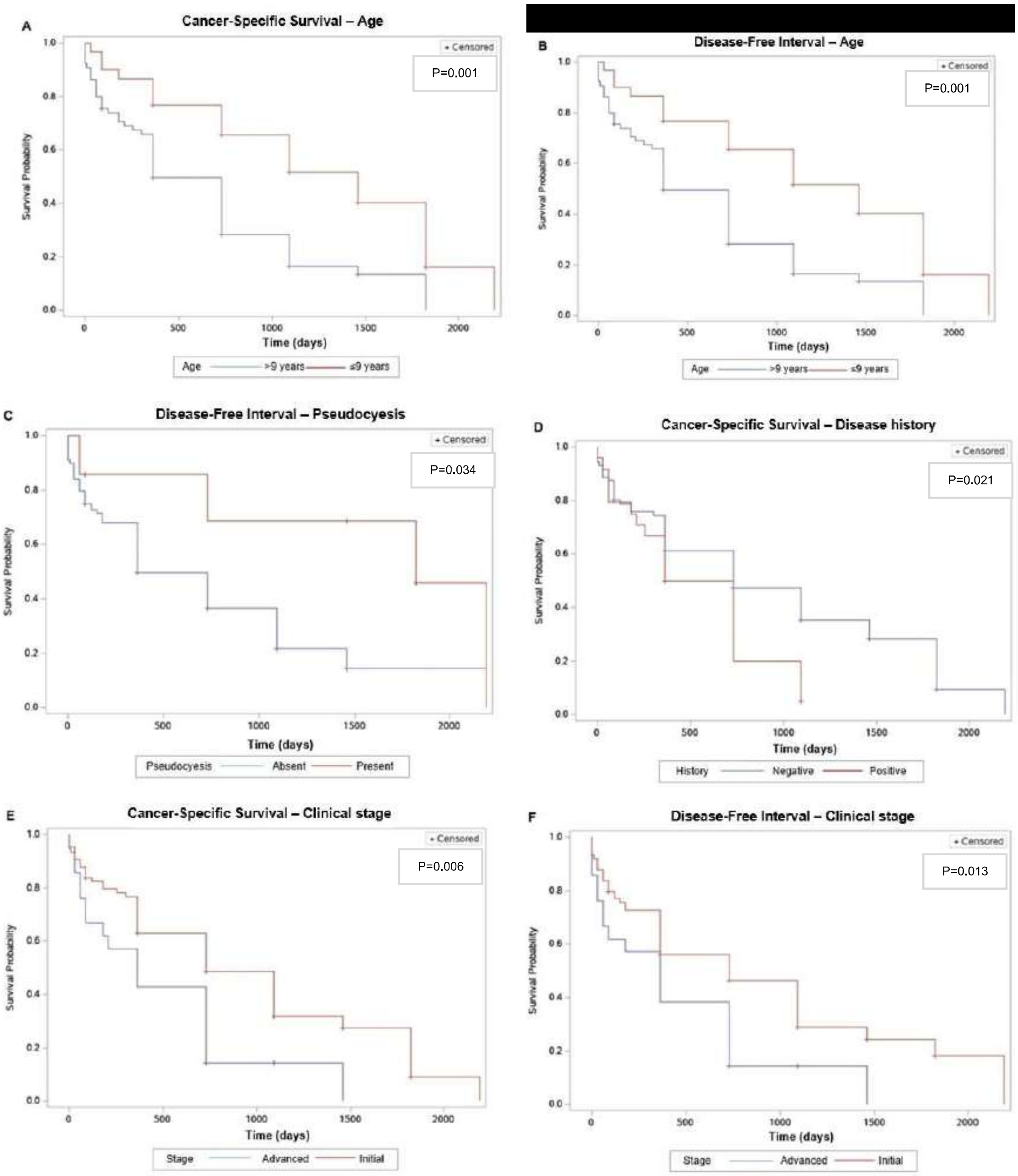
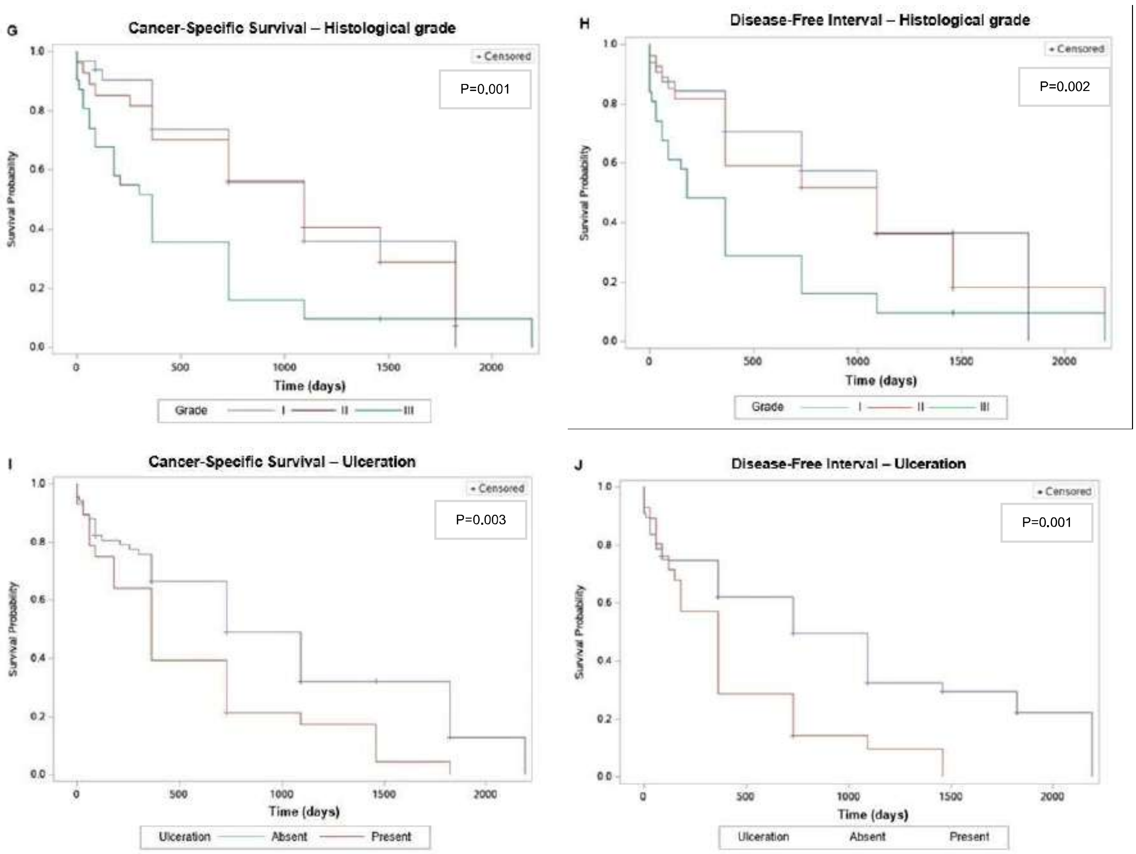
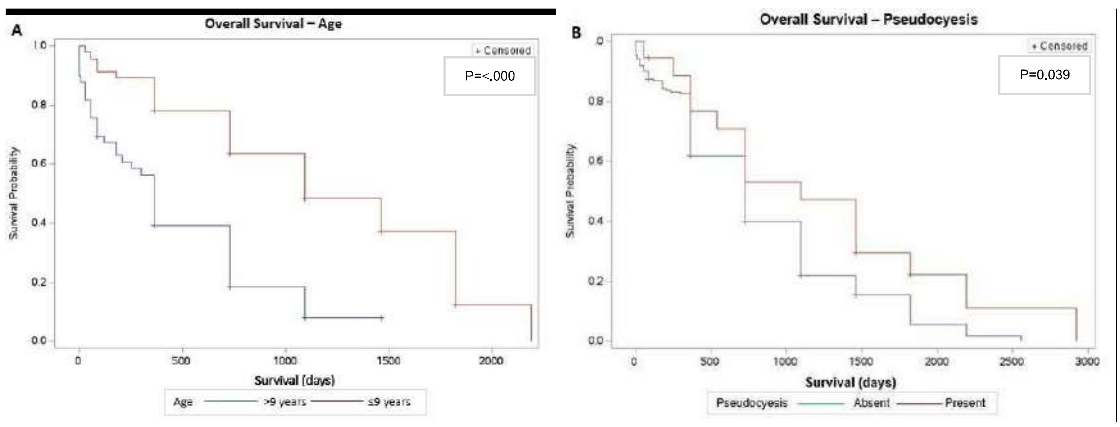
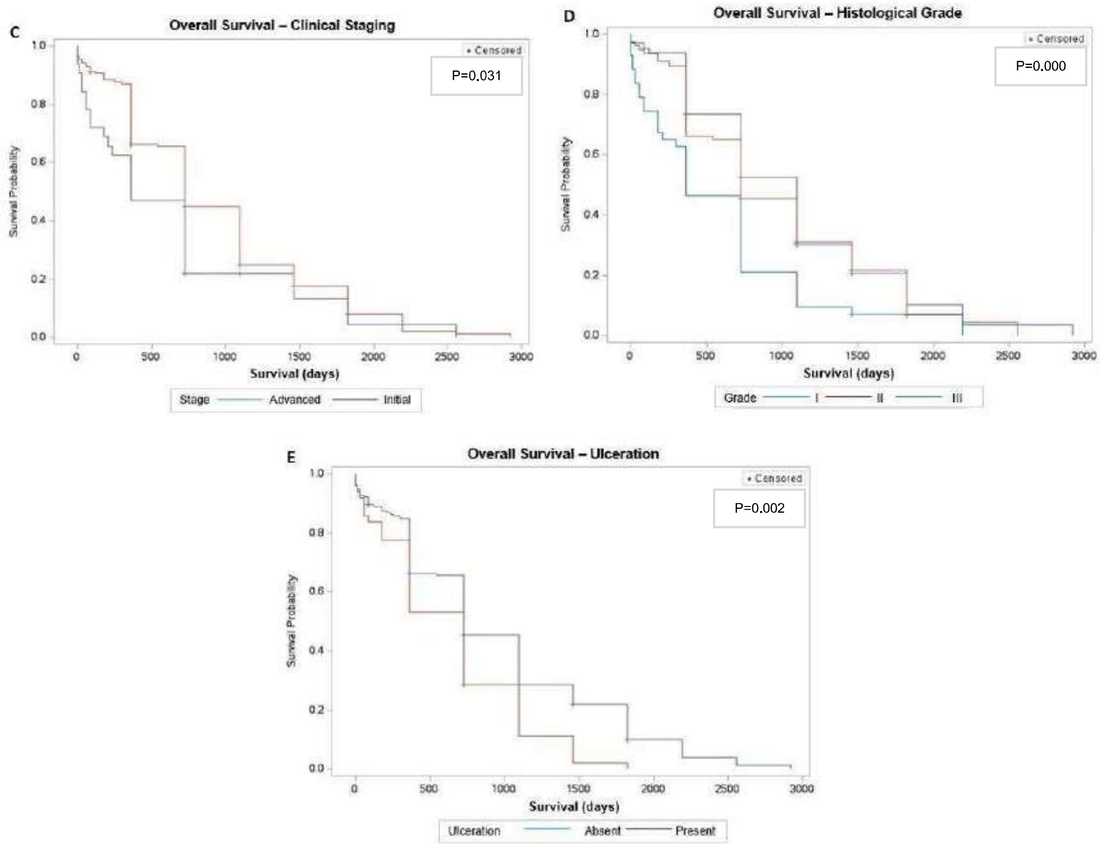
Supplementary materials





Supplementary Figure 1. Cancer-specific survival and disease-free interval curve of Kaplan–Meier of female dogs with malignant mammary neoplasms. Malignant mammary neoplasms classified according to: **A –** CSS age: ≤9 years (median 1.460 days) and >9 years (median 365 days). **B –** DFI age: ≤9 years (median 1.460 days) and >9 years (median 365 days). **C –** DFI pseudocyesis: Present (median 1.825 days) and absent (median 730 days). **D –** CSS disease history: Negative (median 730 days) and positive (median 547 days)**. E –** CSS clinical staging: initial (median 730 days) and advanced (365 days). **F –** DFI clinical staging: initial (median 730 days) and advanced (365 days). **G –** CSS histological grade: **I** (median 1.095 days), **II** (1.095 days) and III (365 days), respectively. **H –** DFI histological grade: **I** (median 1.095 days), II (1.095 days) and III (180 days), respectively. **I –** CSS ulceration: Present (median 365 days) and absent (median 730 days). **J –** DFI ulceration: Present (median 365days) and absent (median 730 days).





Supplementary Figure 2. Overall survival curve of Kaplan–Meier of female dogs with malignant mammary neoplasms. Malignant mammary neoplasms classified according to: **A –** Age: ≤9 years (median 1.095 days) and >9 years (median 365 days). **B –** Pseudocyesis: Present (median 1.095 days) and absent (median 730 days). **C –** Clinical staging: initial (median 730 days) and advanced (365 days). **D –** Histological grade: **I** (median 1.095 days), II (730 days) and III (365 days), respectively. **E** – Ulceration: Present (median 730 days) and absent (median 730 days).

Supplementary Table 1. Histological classification and frequency of mammary tumors diagnosed in female dogs.

Tumors Classification	Histological Classification	N=385	%
1. Non-neoplastic lesions N = 27 (7.01%)	1.1 Adenosis	10	2.60
	1.2 Ductal ectasia	2	0.52
	1.3 Ductal hyperplasia	1	0.26
	1.4 lobular hyperplasia	14	3.64
2. Benign Neoplasms N = 16 (4.16%)	2.1 Adenoma	3	0.78
	2.2 Adenomyoepithelioma	1	0.26
	2.3 Benign mixed tumor	12	3.12
3. Malignant Neoplasms N = 342 (88.83%)	**3.1 Carcinomas**		
	3.1.1 Carcinoma in situ	1	0.26
	3.1.2 Basaloid carcinoma	1	0.26
	3.1.3 Cribriform carcinoma	2	0.52
	3.1.4 Carcinoma in a mixed tumor	169	43.90
	3.1.5 Papillary carcinoma	34	8.83
	3.1.6 Tubular carcinoma	56	14.55
	3.1.7 Solid carcinoma	25	6.49
	3.2 Special type Carcinomas		
	3.2.1 Pleomorphic lobular carcinoma	4	1.04
	3.2.2 Micropapillary carcinoma	17	4.42
	3.2.3 Mucinous carcinoma	1	0.26
	3.2.4 Lipid-rich carcinoma	1	0.26

3.2.5 Secretory carcinoma	1	0.26
3.2.6 Squamous cell carcinoma	2	0.52
3.3 Myoepithelial Neoplasms		
3.3.1 Malignant adenomyoepithelioma	10	2.60
3.3.2 Malignant myoepithelioma	2	0.52
3.4 Sarcomas		
3.4.1 Carcinosarcoma	10	2.60
3.4.2 Chondrosarcoma	3	0.78
3.4.3 Hemangiosarcoma	2	0.52
3.4.4 Osteosarcoma	1	0.26

Supplementary Table 2. Life Survival Estimates and hazard functions in 95 cases with available follow-up data.

	Cancer-specifc survival (*n*=95)										
	Interval		Number Failed	Number Censored	Suvival	Failure	Survival Standard Error	Evaluated At the Midpoint of the Interval			
	Lower	Upper						PDF	PDF Standard Error	Hazard	Hazard Standard Error
Age ≤9.0 years (*n = 30)*	0	500	10	4	1.0000	0	0	0.000455	0.000126	0.000513	0.000161
	500	1000	6	5	0.7727	0.2273	0.0632	0.000314	0.000117	0.000453	0.000184
	1000	1500	8	7	0.6156	0.3844	0.0762	0.000563	0.000162	0.001185	0.0004
	1500	2000	4	1	0.3342	0.6658	0.0842	0.000486	0.000176	0.002286	0.000938
	2000	2500	1	0	0.0911	0.9089	0.0675	0.000182	0.000135	0.004	0
	2500		0	0	0	1.0000	0				
Age >9.0 years (*n = 65*)	0	500	29	3	1.0000	0	0	0.000122	0.000142	0.001758	0.000293
	500	1000	9	1	0.3895	0.6105	0.0708	0.000425	0.000123	0.0015	0.000464
	1000	1500	4	3	0.1770	0.8230	0.0576	0.000258	0.000107	0.002286	0.000938
	1500		0	0	0.0483	0.9517	0.0371				
History of previous disease – Negative (*n = 71*)	0	500	29	5	1.0000	0	0	0.000800	0.000115	0.001	0.00018
	500	1000	9	6	0.6000	0.4000	0.0575	0.000284	0.000087	0.000537	0.000177
	1000	1500	11	9	0.4579	0.5421	0.0603	0.000469	0.000116	0.001375	0.000389
	1500	2000	4	1	0.2236	0.7764	0.0575	0.000325	0.000119	0.002286	0.000938
	2000	2500	1	0	0.0610	0.9390	0.0453	0.000122	0.000091	0.004	0
	2500		0	0	0	1.0000	0				
History of previous disease – Positive (*n =24*)	0	500	10	2	1.0000	0	0	0.00105	0.000229	0.001429	0.000422
	500	1000	6	0	0.4737	0.5263	0.1145	0.000711	0.000225	0.0024	0.000784
	1000	1500	1	1	0.1184	0.8816	0.0780	0.000158	0.000138	0.002	0.001732
	1500		0	0	0.0395	0.9605	0.0525				
Clinical Stage (TNM) Initial (I–III) (*n*= 74)	0	500	27	7	1.0000	0	0	0.000766	0.000116	0.000947	0.000177
	500	1000	9	5	0.6170	0.3830	0.0579	0.000296	0.000090	0.000545	0.00018
	1000	1500	11	9	0.4689	0.5311	0.0615	0.000480	0.000119	0.001375	0.000389
	1500	2000	4	1	0.2290	0.7710	0.0588	0.000333	0.000122	0.002286	0.000938
	2000	2500	1	0	0.0625	0.9375	0.0464	0.000125	0.000093	0.004	0
	2500		0	0	0	1.0000	0				
Clinical Stage (TNM) Advanced (IV–V) (*n*=21)	0	500	12	0	1.0000	0	0	0.00114	0.000216	0.0016	0.000423
	500	1000	6	1	0.4286	0.5714	0.1080	0.000605	0.000203	0.002182	0.000747
	1000	1500	1	1	0.1261	0.8739	0.0741	0.000168	0.000139	0.002	0.001732
	1500		0	0	0.0420	0.9580	0.0544				
Histological grade **(*n* = 90)** Grade I (*n*= 32)	0	500	8	7	1.0000	0	0	0.000561	0.000168	0.000653	0.000228
	500	1000	4	2	0.7193	0.2807	0.0842	0.000360	0.000161	0.000571	0.000283
	1000	1500	4	6	0.5395	0.4605	0.1002	0.000539	0.000215	0.001333	0.000629
	1500	2000	1	0	0.2697	0.7303	0.1077	0.000539	0.000215	0.004	0
	2000		0	0	0	1.0000	0				

Histological	0	500	8	0	1.0000	0	0	0.000593	0.000176	0.000696	0.000242
grade	500	1000	4	4	0.7037	0.2963	0.0879	0.000331	0.000151	0.000533	0.000264
(*n* = 90)	1000	1500	5	2	0.5381	0.4619	0.0988	0.000538	0.000197	0.001333	0.000562
	1500	2000	3	1	0.2691	0.7309	0.0984	0.000461	0.000196	0.003	0.001146
Grade II	2000		0	0	0.0384	0.9616	0.0523				
(*n*= 27)											
	0	500	20	0	1.0000	0	0	0.00129	0.000172	0.001905	0.000375
Histological	500	1000	6	0	0.3548	0.6452	0.0859	0.000387	0.000142	0.0015	0.000568
grade	1000	1500	2	2	0.1613	0.8387	0.8387	0.000161	0.000104	0.001333	0.000889
(*n* = 90)	1500	2000	0	0	0.0806	0.9194	0.9194			0	
	2000	2500	1	0	0.0806	0.9194	0.9194	0.000161	0.000104	0.004	0
Grade III	2500		0	0	0	1.0000	1.0000				
(*n*= 31)											
	0	500	22	7	1.0000	0	0	0.000693	0.000119	0.000838	0.000175
	500	1000	10	5	0.6535	0.3465	0.0597	0.000368	0.000104	0.000104	0.000205
Ulceration –	1000	1500	8	10	0.4694	0.5306	0.0654	0.000417	0.000124	0.001143	0.000387
Absent	1500	2000	3	1	0.2608	0.7392	0.0659	0.000348	0.000145	0.002	0.001
(*n* = 67)	2000	2500	1	0	0.9131	0.9131	0.0620	0.000174	0.000124	0.004	0
	2500		0	0	0	1.0000	0				
	0	500	17	0	1.0000	0	0	0.00121	0.000185	0.001744	0.000381
Ulceration –	500	1000	5	1	0.3929	0.6071	0.0923	0.000374	0.000150	0.00125	0.000531
Present	1000	1500	4	0	0.2058	0.7942	0.0775	0.000329	0.000144	0.002667	0.000994
(*n* = 28)	1500	2000	1	0	0.0412	0.9588	0.0399	0.000082	0.000080	0.004	0
	2000		0	0	0	1.0000	0				
Disease-free interval (*n*=95)											
	0	500	12	3	1.0000	0	0	0.000539	0.000133	0.000623	0.000178
	500	1000	6	5	0.7303	0.2697	0.0665	0.000308	0.000115	0.000471	0.000191
Age ≤9.0	1000	1500	9	7	0.5766	0.4234	0.0766	0.000629	0.000164	0.0015	0.000464
years	1500	2000	1	2	0.2621	0.7379	0.0788	0.000175	0.000152	0.0008	0.000784
(*n* = 30)	2000	2500	1	0	0.1747	0.8253	0.0886	0.000349	0.000177	0.004	0
	2500		0	0	0	1.0000	0				
	0	500	30	4	1.0000	0	0	0.000128	0.000140	0.001875	0.000302
	500	1000	6	0	0.3617	0.6383	0.0701	0.000289	0.000107	0.001	0.000395
Age >9.0	1000	1500	5	3	0.2170	0.7830	0.0621	0.000289	0.000112	0.002	0.000775
years	1500	2000	0	0	0.0723	0.9277	0.0427	0		0	
(*n* = 65)	2000	2500	1	0	0.0723	0.9277	0.0427	0.000145	0.000085	0.004	0
	2500		0	0	0	0	0				
	0	500	41	6	1.0000	0	0	0.000965	0.000108	0.001271	0.000188
Pseudocyesis	500	1000	11	5	0.5176	0.4824	0.0542	0.000296	0.000081	0.000667	0.000198
–Absent	1000	1500	14	9	0.3697	0.6303	0.0540	0.000505	0.000106	0.002074	0.000474
(*n* = 88)	1500	2000	0	1	0.1172	0.8828	0.0417	0		0	
	2000	2500	1	0	0.1172	0.8828	0.0417	0.000234	0.000083	0.004	0
	2500		0	0	0	1.0000	0				
	0	500	1	1	1.0000	0	0	0.000308	0.000283	0.000333	0.000332
	500	1000	1	0	0.8462	0.1538	0.1415	0.000338	0.000308	0.000444	0.000442
Pseudocyesis	1000	1500	0	1	0.6769	0.3231	0.1890	0		0	
–Present	1500	2000	1	1	0.6769	0.3231	0.1890	0.000542	0.000446	0.001	0.000968
(*n* = 7)	2000	2500	1	0	0.4062	0.5938	0.2384	0.000812	0.000477	0.004	0
	2500		0	0	0	1.0000	0				
	0	500	29	7	1.0000	0	0	0.000823	0.000117	0.001036	0.000186
Clinical Stage	500	1000	7	4	0.5887	0.4113	0.0586	0.000229	0.000081	0.000431	0.000162
(TNM)	1000	1500	13	9	0.4742	0.5258	0.0611	0.000548	0.000121	0.001625	0.000412
Initial (I–III)	1500	2000	1	2	0.2002	0.7998	0.0557	0.000100	0.000091	0.000571	0.000566
(*n*= 74)	2000	2500	2	0	0.1502	0.8498	0.0602	0.000300	0.000120	0.004	0
	2500		0	0	0	1.0000	0				
Clinical Stage	0	500	13	0	1.0000	0	0	0.00124	0.000212	0.001793	0.000445
(TNM)	500	1000	5	1	0.3810	0.6190	0.1060	0.000508	0.000193	0.002	0.000775
	1000	1500	1	1	0.1270	0.8730	0.0745	0.000169	0.000139	0.002	0.001732

Advanced	1500		0	0	0.0423	0.9577	0.0548				
(IV–V)											
(*n*=21)											
Histological	0	500	6	6	1.0000	0	0	0.000414	0.000150	0.000462	0.000187
grade	500	1000	4	3	0.7931	0.2069	0.0752	0.000343	0.000155	0.000485	0.000241
(*n* = 90)	1000	1500	6	6	0.6216	0.3784	0.0961	0.000746	0.000224	0.001714	0.000632
	1500	2000	1	0	0.2486	0.7514	0.1037	0.000497	0.000207	0.004	0
Grade I	2000		0	0	0	1.0000	0				
(*n*= 32)											
	0	500	11	1	1.0000	0	0	0.000830	0.000191	0.001048	0.000305
Histological	500	1000	2	2	0.5849	0.4151	0.0957	0.000167	0.000113	0.000308	0.000217
grade	1000	1500	6	2	0.5013	0.4987	0.0986	0.000602	0.000195	0.001714	0.000632
(*n* = 90)	1500	2000	0	2	0.2005	0.7995	0.0871	0		0	
	2000	2500	1	0	0.2005	0.7995	0.0871	0.000401	0.000174	0.004	0
Grade II	2500		0	0	0	1.0000	0				
(*n*= 27)											
	0	500	22	0	1.0000	0	0	0.00142	0.000163	0.0022	0.000392
Histological	500	1000	4	0	0.2903	0.7097	0.0815	0.000258	0.000120	0.001143	0.000548
grade	1000	1500	2	2	0.1613	0.8387	0.0661	0.000161	0.000104	0.001333	0.000889
(*n* = 90)	1500	2000	0	0	0.0806	0.9194	0.0521			0	
	2000	2500	1	0	0.0806	0.9194	0.0521	0.000161	0.000104	0.004	0
Grade III	2500		0	0	0	1.0000	0				
(*n*= 31)											
	0	500	23	7	1.0000	0	0	0.000724	0.000121	0.000885	0.00018
	500	1000	8	4	0.6378	0.3622	0.0603	0.000292	0.000095	0.000516	0.000181
Ulceration –	1000	1500	10	10	0.4920	0.5080	0.0649	0.000492	0.000128	0.001333	0.000398
Absent	1500	2000	1	2	0.2460	0.7540	0.0639	0.000123	0.000111	0.000571	0.000566
(*n* = 67)	2000	2500	2	0	0.1845	0.8155	0.0716	0.000369	0.000143	0.004	0
	2500		0	0	0	1.0000	0				
	0	500	19	0	1.0000	0	0	0.00136	0.000177	0.002054	0.000404
Ulceration –	500	1000	4	1	0.3214	0.6786	0.0883	0.000303	0.000138	0.001231	0.000586
Present	1000	1500	4	0	0.1702	0.8298	0.0722	0.000340	0.000144	0.004	0
(*n* = 28)	1500		0	0	0	1.0000	0				

3. CAPÌTULO: 2

PLATELET COUNT AND MCHC AS INDEPENDENT PROGNOSTIC MARKERS FOR FELINE MAMMARY CARCINOMAS

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Abstract

Mammary neoplasms are common in felines species and represent a significant disease for its unfavorable prognosis. Changes in the blood count and serum biochemical profile of these patients have potential as non-invasive prognostic markers prior to mastectomy, however, they are poorly described in literature. In this study univariate and multivariate analyses were performed using these factors to determine the effect of each parameter on the one-year survival time after the surgical procedure in these animals. The median overall survival (OS) and the disease-free survival (DFS) were 365 and 242 days, respectively. In univariate analysis, values within the reference range of monocyte, platelet and creatinine counts were identified as significant prognostic factors for OS and only creatinine was significant for DFS ($P<0.05$). In the multivariate analysis, platelets and mean corpuscular hemoglobin concentration (MCHC) remained independent prognostic factors for OS. The results presented suggest that monocytes, platelets and creatinine may be important non-invasive pre-surgical prognostic markers, and that platelet count and MCHC are independent prognostic markers for feline mammary carcinomas (FMC). The correlation between such alterations is of important relevance for veterinary oncology, and prospective studies are needed to validate their clinical use and that platelet count and MCHC are independent prognostic markers for FMC. The results found in this study can also be studied in human medicine, regarding blood markers in human breast cancer (HBC).

Keywords: biomarkers, cat mammary tumors, predictive factors, mean corpuscular hemoglobin concentration, overall survival

3.1. Introduction

Breast cancer is the most diagnosed neoplasm among women (Soare and Soare, 2019) and feline mammary carcinoma (FMC) is the third most common tumor type in cats (Morris, 2013; Giménez et al., 2010), 80-90% of feline mammary tumors are malignant (Giménez et al., 2010; Lana, Rutteman and Withrow, 2007). Like human breast cancer (HBC) (Padmanabhan et al., 2020), FMC presents aggressive biological behavior (Hassan et al., 2017) with infiltration and systemic progression (Petrucci et al., 2021; Seixas et al., 2011; Giménez et al., 2010), representing the most common cause of death in middle-aged and older cats (Petrucci et al., 2020a; Giménez et al., 2010). Aggressive surgery via radical mastectomy remains the treatment of choice in cats (Petrucci et al, 2020b; Gemignani et al., 2018), as adjuvant chemotherapy is not helpful in some cases (Michishita et al., 2016).

Thus, the identification of new diagnostic biomarkers is necessary, not only to improve the clinical outcome of FMC, but also because cats with FMC share epidemiological, clinicopathological and histopathological characteristics, as well as in carcinogenesis, with HBC (Padmanabhan et al., 2020; Urbano et al., 2020; Hassan et al., 2017; Cannon, 2015; Caliari et al., 2014).

The prognosis is poor for most cats with carcinomas (Zappulli et al., 2015), OS is heavily dependent on several clinical and histological factors (Dagher et al., 2019; Seixas et al., 2011). Tumor microenvironment, inflammation, and immune response play important roles in tumor progression and prognosis (Fridman et al, 2017; Hanahan and Weinberg, 2011). Since several hematological, molecular and serum markers are associated with prognosis (Nascimento et al., 2020; Marques et al., 2017; Soares *et al*., 2016; Giménez et al., 2010), there is great interest in establishing predictive and prognostic hematological markers in veterinary oncology (Chiti et al., 2019; Henriques et al., 2021).

The absolute leukocyte count and the neutrophil/lymphocyte ratio (NLR) have demonstrated prognostic information in dogs with lymphoma, osteosarcoma, mast cell tumors, and soft tissue sarcomas (Macfarlane et al., 2016a; Macfarlane et al., 2016b; Marconato et al., 2015; Sottnik et al, 2010) and in cats with mammary carcinoma (Petrucci et al., 2021). Neutrophils and platelets are involved in tumor occurrence, proliferation and metastasis (Contursi et al., 2018; Coffelt, Wellenstein and De Visser, 2016).

Serum markers related to renal dysfunction are already being used in cancer patients to predict mortality. Creatinine and serum albumin (ALB) have been investigated in human patients as prognostic parameters in liposarcoma, where a low albumin and a high albumin-to-creatinine ratio independently predict poor overall survival (Panotopoulos et al., 2016), myofibroblastic and soft tissue fibroblastic sarcomas (Willegger et al., 2017), colorectal carcinoma (Giessen-Jung et al., 2015), upper urinary tract urothelial carcinoma (Kim et al., 2015), prostate carcinoma (Tollefson et al., 2013), multiple myeloma (Chen et al., 2015) and epithelial ovarian cancer, where a serum creatinine level of 1.2 mg/dL or higher is associated with poor survival (Lafleur et al., 2018).

Although complete blood count (CBC) and of biochemilcal parameters are routinely available, the role of these hematological parameters as prognostic markers has never been investigated in FMC or HBC. The aim of this study was to evaluate the impact of the pre-surgical CBC variables, erythrocytes indices ((MCV – Mean Corpuscular Volume. MCH – Mean Corpuscular Hemoglobin and MCHC – Mean Corpuscular Hemoglobin Concentration) and the of biochemilcal parameters as potential prognostic factors for overall survival (OS) and disease-free survival (DFS) of FMC.

3.2. Materials and methods

3.2.1. Case selection

The clinical database of the Veterinary Hospital of the Federal University of Viçosa (HVT–UFV) was reviewed between January 2012 and May 2021 for female cats with history of mastectomy and histological diagnosis of mammary carcinoma. All procedures were performed according to the Ethics Committee for the Use of Animals (CEUA/UFV), protocol 18/2020.

The cases were classified according to the TNM staging (size, lymph node involvement and distant metastases), proposed by the World Health Organization (WHO). A complete medical record available with pre-surgical hematological data (within 10 days before surgery) was mandatory for inclusion. Cats were excluded if they met one of the following criteria: (a) had received blood transfusions or had undergone surgical procedures within the previous three months; (b) were positive for feline immunodeficiency virus and/or feline leukemia virus infection;

(c) received an immunosuppressive or immunostimulating treatments within two months prior to blood sample collection.

All staging procedures were performed before surgery and consisted of chest radiographs in ventrodorsal and right and left lateral views and abdominal ultrasound, complete pre-surgical hematological and biochemical profiles. The treatment performed on the animals was exclusively surgical resection. The blood sample was collected by external jugular venipuncture and was put in tubes containing 10% Ethylenediamine Tetra-acetic Acid (EDTA) for hematological parameters and in dry tubes with separator gel for biochemical parameters. The serum was separated by centrifugation for 10 minutes at 3500 rpm (rotations per minute). The collected samples were processed in the Clinical Analysis Laboratory of the Department of Veterinary Medicine – UFV.

The hematological parameters analyzed were total leukocytes, neutrophils, lymphocytes, eosinophils, monocytes, red blood cells (RBC), hemoglobin (Hb), hematocrit (Ht), erythrocytes indices (MCV, MCH and MCHC) and platelet count. These parameters were analyzed using the hematology analyzer model HumaStar 300, Human brand.

The biochemical paorameters of glutamic-pyruvic transaminase (GPT), alkaline phosphatase (ALP), glutamic-oxaloacetic transaminase (GOT), total plasma protein (TPP), glucose, ALB, globulin, urea and creatinine were analyzed using the automated HumaCount Plus instrument, Human brand, with commercial kits. Reference intervals for blood count and biochemistry according to Thrall et al. (2015) are described in Table 1. The NLR was calculated as the ratio between absolute neutrophils and lymphocytes.

Tumors were classified according to the consensus for the diagnosis, prognosis and treatment of FMC (Cassali et al., 2020) and evaluated according to the Nottingham System classification[19]. The worst prognosis lesion was selected in animals with multiple tumors.

3.2.2. Follow-up and clinical outcome

After surgery, patients were followed up every six months for a minimum period of 12 months and owners were contacted by telephone in cases of loss of clinical follow-up. DFS was defined as the period between the surgery and development of local recurrence and/or regional or distant metastasis. OS was defined as the period between surgical excision of the tumor and tumor-related death. Animals whose death occurred from unknown causes and with lost follow-up were not considered. Finally, the animals were analyzed as to whether the blood count and biochemical results were within the reference value (Into Group) or outside the reference value (Out Group).

3.2.3. Statistical analysis

For data analysis the Statistical Analysis System (SAS OnDemand) was used. Quantitative variables were analyzed by Bartlett test and Kolmogorov-Smirnov test to evaluate homogeneity of variances and normality of errors, respectively. Data from ALP and monocytes were submitted to logarithmic transformation ($y' = \log(y+1)$), data from lymphocytes, monocytes, and neutrophils (segmented): lymphocytes ratio were submitted to square root transformation (($y' = \sqrt{y+0.5}$). Data weas then submitted to analysis of variance (GLM Procedure) according to the model:

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where: Y_{ij}, response; μ , constant; G_i, effect on malignant neoplasms; e_{ij}, error.

The OS time and the DFS were analyzed by survival analysis (Lifetest Procedure) and the comparisons among the strata were performed by Log-Rank test. Significance adopted level was 0.05.

3.3. Results

Fourty cases in the database had histological diagnosis of mammary carcinoma. Twenty-two cats did not meet the inclusion criteria and were excluded. Eighteen cats met the inclusion criteria. Regarding blood count, biochemistry and NLR data, the results are presented in Table 1.

The analysis of erythrogram revealed 5.56% of cats (1/18) with normocytic and normochromic, non-regenerative anemia.

The analysis of leukogram revealed that 22.22% (4/18) of the cats had leukopenia, 22.22% (4/18) leukocytosis and 38.88% (7/18) neutrophilia. Regarding lymphocytes, 55.56% (10/18) had lymphopenia and 44.44% (8/18) lymphocytosis. As for monocytes, only 11.11% (2/18) had monocytosis.

Eosinophilia was observed in 16.67% (3/18) and thrombocytopenia in 16.67% (3/18) of cases. The mean NLR was 7.48 and the median was 4.53.

Regarding the biochemical analysis, the concentration of ALP was increased in 22.22% (4/18; 21–301 U/L) of the cats, while in 5.56% (1/18; 21–301 U/L) it was below the reference values. Quantitation of GPT was elevated compared to normal serum levels in 27.78% (5/18; 9–329.7 U/L) of the animals. The number of cats with high levels of GOT, represented 88.89% (16/18; 30–148 U/L) of the cases. Serum glucose levels were increased in 27.78% (5/18) of the cats and reduced in 5.56% (1/18; 71–188 mg/dL). ALB was normal in the 18 (2.3–3.27 g/dL) animals, and globulin was elevated in 27.78% (5/18; 3.66–6.22 g/dL). Creatinine values were below the reference in 5.56% (1/18; 0.5–1.71 mg/dL) of the cats. Of the animals analyzed, 11.11% (2/18; Urea 40–138 mg/dL) had uremia.

Table 1. Hematological and biochemistry parameters of cats diagnosed with mammary carcinoma.

Parameters	Mean±SD	Reference interval[a]
RBC (10^6/µL)	7.2 ± 1.5	5.0 – 10.0
Hb (g/dL)	10.9 ± 2.0	8 – 15
Ht (%)	32.9 ± 6.4	24 – 45
MCV (fL)	46.4 ± 4.9	39 – 55
MCH (pg)	14.7 ± 1.8	12.5 – 17.5
MCHC (g/dL)	33.1 ± 1.7	30 – 36
Total leukocytes (µL)	13.4 ± 6.5	5.500 – 19.500
Neutrophils (%)	71.5 ± 10.3	35 – 75
Immature neutrophils (%)	2.1 ± 4.8	0 – 3
Lymphocytes (%)	17.3 ± 10.1	7.69 – 35.90
Monocytes (%)	2.7 ± 3.0	0 – 15.45
Eosinophils (%)	6.0 ± 3.2	0 – 27.27
Platelets (µL)	230.1 ± 100.9	150.000 – 700.000
NLR	7.5	-
ALP (U/L)	67.5 ± 70.5	25 – 93
GPT (U/L)	76.4 ± 77.7	6 – 83
GOT (U/L)	65.3 ± 31.0	26 – 43
TPP (g/dL)	7.5 ± 0.7	5.4 – 7.8
Creatinine (mg/dL)	1.3 ± 0.3	0.8 – 1.8
Urea (mg/dL)	56.5 ± 22.5	42.8 – 64.2
Glucose (mg/dL)	120.2 ± 34.7	73 – 134
Albumine (g/dL)	2.8 ± 0.3	2.1 – 3.3
Globuline (g/dL)	4.7 ± 0.7	2.6 – 5.1

Mean ± standard deviation. **RBC –** Red Blood Cells. **Hb –** Hemoglobin. **Ht –** Hematocrit. **MCV –** Mean Corpuscular Volume. **MCH –** Mean Corpuscular Hemoglobin. **MCHC –** Mean Corpuscular Hemoglobin Concentration. **ALP –** Alkaline phophatase. **GPT –** Glutamic-Pyruvic Transaminase. **GOT –** Glutamic-Oxalacetic Transaminase. **TPP –** Total Plasma Protein. **(**Thrall et al., 2015).

Solid carcinoma and papillary carcinoma were the most common histological types 27.78% (5/18), followed by tubular carcinoma 11.11% (2/18). Among the special types of carcinoma, the micropapillary 16.67% (3/18) was the most observed. Carcinosarcoma was the only histological type found within the sarcoma classification, present in 5.56% (1/18) of the animals (Table 2).

Table 2. Histological classification of affected feline mammary carcinomas (n = 18).

Tumor Classification	Histological Classification	N	%
	1.1 Carcinomas		
	1.1.1 Cribriform carcinoma	1	5.56
	1.1.2 Papillar carcinoma	5	27.78
	1.1.3 Tubular carcinoma	2	11.11
1. Carcinomas	1.1.4 Solid carcinoma	5	27.78
N = 18 (100%)	**1.2 Special type Carcinomas**		
	1.2.1 Micropapillary carcinoma	3	16.67
	1.2.2 Mucinous carcinoma	1	5.56
	1.3 Sarcomas		
	1.3.1 Carcinosarcoma	1	5.56

3.3.1. Analysis of prognostic variables

The median OS was 365 days and the median DFS was 242 days (Figure 1A and 1B, respectively).

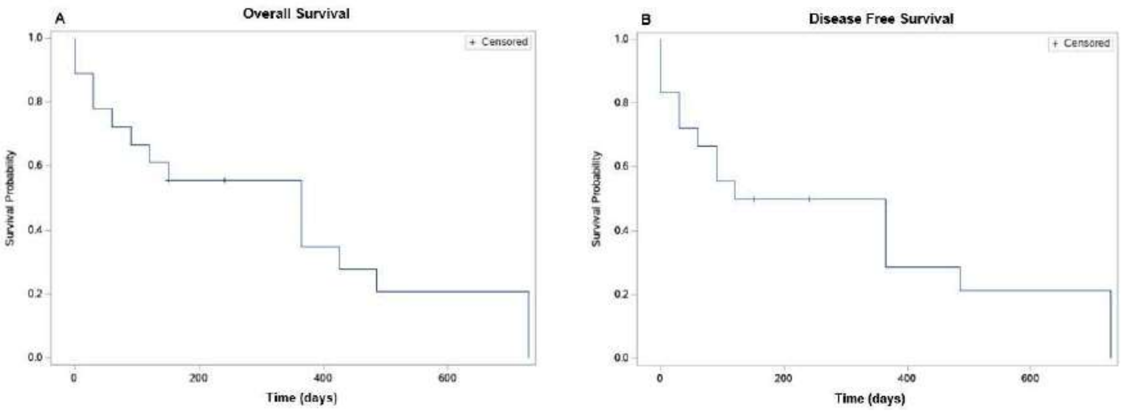

Figure 1. Kaplan-Meier curves for: A – Overall Survival (OS) of 18 cats. The median for OS was 365 days. B – Disease-free period (DFS) of 18 cats. The median for DFS was 242 days.

No significant association was observed between NLR, OS and DFS. However, patients with higher NLR had lower OS and DFS and OS and DFS were below the median.

The statistically significant factors for OS were: monocytes (P=0.0222), platelets (P= 0.0453) and creatinine (P=0.0047) (Table 3). Only creatinine showed statistical correlation with DFS (P=0.0253; Table 4). More details of the univariate analysis foror OS and DFS are presented in Table 3 and Table 4, respectively.

Table 3. Univariate Cox proportional hazard models for overall survival in 18 cats[a]

	Overall Survival (*n*=18)						
	Group Into			Group Out			P
	n	Median survival (days)	Average 1-year survival rate *n* (%)	*n*	Median survival (days)	Average 1-year survival rate *n* (%)	
RBC	16	365	4 (25)	2	380	0	0.5821
Hb	16	365	4 (25)	2	380	0	0.5821
Ht	16	365	4 (25)	2	380	0	0.5821
MCV	17	365	4 (23.53)	1	485	0	0.7128
MCH	15	365	4 (26.67)	3	365	1 (33.33)	0.6829
MCHC	16	257	4 (25)	2	730	0	0.1142
Total leukocytes	10	365	3 (30)	8	242	2 (25)	0.3133
Neutrophilis	11	365	3 (27.27)	7	150	2 (28.57)	0.1720
Lymphocytes	7	90	1 (14.29)	11	365	4 (36.36)	0.5426
Immature neutrophils	15	150	3 (20)	3	485	1 (33.33)	0.2588
Monocytes	16	365	5 (31.25)	2	45	0	0.0222
Eosinoplils	15	365	4 (26.67)	3	-[b]	0	0.1106
Platelets	15	365	5 (33.33)	3	60	0	0.0453
ALP	13	365	4 (30.77)	5	365	1 (20)	0.7649
GPT	13	150	4 (30.77)	5	365	1 (20)	0.4254
GOT	2	-[c]	0	16	365	5 (31.25)	0.9031
TPP	14	257	3 (21.43)	4	425	2 (50)	0.5949
Creatinine	17	365	5 (29.41)	1	0	0	0.0047
Urea	12	365	4 (33.33)	6	90	0	0.1838
Glucose	12	257	3 (25)	6	365	2 (33.33)	0.6277
Albumine	18	365	5 (27.78)	0	0	0	-
Globuline	13	365	3 (23.08)	5	365	2 (40)	0.9395

[a]Survival values are percentages for cumulative survival determined by Log-Rank test. Significance level adopted was <0.05.

[b]Mean survival: 141 days.

[C]Mean survival: 150 days.

Table 4. Univariate Cox proportional hazard models for disease-free survival in 18 cats[a]

	Disease-free survival (*n*=18)						
	Group Into			Group Out			P
	n	Median survival (days)	Average 1-year survival rate *n* (%)	*n*	Median survival (days)	Average 1-year survival rate	

						n (%)	
RBC	16	242	3 (18.75)	2	380	0	0.5714
Hb	16	242	3 (18.75)	2	380	0	0.5714
Ht	16	242	3 (18.75)	2	380	0	0.5714
MCV	17	120	3 (17.65)	1	485	0	0.7457
MCH	15	120	3 (20)	3	365	1 (33.33)	0.6051
MCHC	16	105	3 (18.75)	2	730	0	0.1027
Total leukocytes	10	287	2 (20)	8	242	1 (12.5)	0.4714
Neutrophilis	11	365	3 (27.27)	7	90	1 (14.29)	0.0633
Lymphocytes	7	60	0	11	365	3 (27.27)	0.3661
Immature neutrophils	15	90	2 (13.33)	3	485	1 (33.33)	0.2399
Monocytes	16	365	4 (25)	2	45	0	0.0923
Eosinoplils	15	365	4 (26.67)	3	-[b]	0	0.1158
Platelets	15	365	4 (26.67)	3	60	0	0.0656
ALP	13	90	3 (23.08)	5	365	1 (20)	0.5798
GPT	13	90	3 (23.08)	5	365	1 (20)	0.2721
GOT	2	-[c]	0	16	242	4 (25)	0.7239
TPP	14	105	2 (14.29)	4	425	2 (50)	0.5536
Creatinine	17	365	4 (23.53)	1	0	0	0.0253
Urea	12	365	3 (25)	6	75	0	0.2610
Glucose	12	105	2 (16.67)	6	365	2 (33.33)	0.5245
Albumine	18	242	4 (22.22)	0	0	0	-
Globuline	13	120	2 (15.38)	5	365	2 (40)	0.8666

[a]Survival values are percentages for cumulative survival determined by Log-Rank test. Significance level adopted was <0.05.

[b]Mean survival: 121 days.

[c]Mean survival: 90 days.

The type of procedure performed (radical mastectomy, lumpectomy or regional mastectomy) and the histological type of FMC were not significant for OS and DFS (Table 5). The multivariate analysis of Cox proportional hazards for OS is shown in Table 6. Only platelet count (P=0.0467) and MCHC (P=0.0817) were maintained in the final model (P<0.10). Multivariate analysis showed that MCHC and platelet count were independent prognostic factors for OS.

Table 5. One-year corrected survival rates for procedure performed and histological type[a]

		Overall survival (*n*=18)			**Disease-free survival** (*n*=18)		
		n	Median survival (days)	Average 1-year survival rate *n* (%)	*n*	Median survival (days)	Average 1-year survival rate *n* (%)
Histological type (*n*=13)	Micropapillary carcinoma	3	365	1 (33.33)	3	60	0
	Papillary carcinoma	5	365	2 (40)	5	365	5 (100)
	Solid carcinoma	5	30	0	5	30	0
	P		0.7503			0.5830	
Surgical technique	Radical mastectomy	13	365	4 (30.77)	13	365	3 (23.08)
	Lumpectomy and Regional mastectomy	5	60	1 (20)	5	60	1 (20)
	P		0.4147			0.5281	

[a]Survival values are percentages for cumulative survival determined by Log-Rank test. Significance level adopted was <0.05.

Table 6. Analysis of maximum likelihood estimates of multivariate logistic regression to predict the probability of overall survival for at least 1 year after surgery in cats affected by mammary tumor.

Parameter	Estimate	Standard Error
Intercept	-72.8786	40.1219
MCHC	1.9740	1.1340
Platelets	0.000037	0.000018

c (Area under ROC curve) = 0.922

3.4. Discussion

Hematological parameters, which may reflect the immunological status, are of great importance for the survival and prognosis of cancer patients. To the best of our knowledge, this is the first study investigating the impact of the pre-surgical level of CBC, erythrocyte indices (MCV, MCH and MCHC) and serum biochemistry profile of FMC. Our results demonstrate that MCHC and platelet count are independent predictive prognostic markers for OS in cats diagnosed with mammary carcinoma.

Systemic inflammation is recognized as a hallmark of cancer development (Cullinane et al, 2020) and the haematological level of neutrophils, lymphocytes, monocytes and leukocytes is used as a prognostic marker in several types of human cancers (Corbeau, Jacot and Guiu, 2020; Grenader et al., 2016; Gu et al., 2015). Neutrophils are the first line of innate immunity and a major source of cytokines and tumor growth promoting factors (Howard, Kanetsky and Egan, 2019; Shaul et al., 2018), contribute to angiogenesis, metastasis, inhibition of apoptosis and tumor progression (Rimini et al., 2020; Masucci et al., 2019). Lymphocytes are crucial for effective antitumor response (Wang et al., 2016; Galon et al., 2013) due to the CD3 receptor which is a costimulator that initiates a signaling cascade. After antigen recognition CD3 promotes cell activation, proliferation and survival, leading to a good prognostic outcome in breast cancer (Krasniqi et al., 2019; Allaoui et al., 2017). Importantly, neutrophilia inhibits the cytotoxic activity of immune cells such as lymphocytes, natural killer cells and T cells that would act the antitumor immune response (Rotondo et al., 2011). In the present study, neutrophils did not interfere with OS, they just reduced the DFS of animals with abnormal neutrophil count (Table 4) when compared to those where neutrophils were within the reference value.

In our study, monocytosis was observed in two patients and both had necrosis described in the histopathological diagnosis. According to Childress (Childress, 2012), monocytosis is characteristic of chronic inflammatory processes and the moderate increase in the number of monocytes, associated or not with neutrophilia, is observed in oncological patients, especially in whom that have lesions with necrotic areas. Macrophages contribute to tumor growth and escape by secreting cytokines. Monocytes can differentiate into macrophages when they enter tissue, aiding tumor progression (Diaz-Montero, Finke and Montero et al., 2014) this corroborates our findings where abnormal monocyte count was a significant prognostic factor for OS. The leukocytosis observed in this study probably reflects the inflammatory condition resulting from breast cancer (Childress, 2012).

In the present study, NLR was not a prognostic factor for OS and DFS. However, higher levels of NLR were associated with increased risk of tumor-related death by Koh et al. (2015) in HBC, and by Naito et al. (2021) and Petrucci et al. (2021) in FMC. Furthermore, lower NLR is associated with higher OS and DFS in HBC (Rimini et al., 2020). There is still no established standard reference value for NRL, however, our results showed higher values than Giménez et al. (2010) and Petrucci et al. (2021). In addition, lymphopenia has been associated with inhibition of cancer immunosurveillance. In the early stages of tumor progression, dendritic cells suppress tumor growth and induce the immunoactive phase. However, dendritic cells change their properties under the influence of neoplastic cell, affecting regulatory T cells, that suppress specific antitumor T lymphocytes (Scarlett et al., 2012). These changes promote a relative decrease in the total number of lymphocytes, thus suggesting that a high NLR may suggest increased tumor growth and inhibition of tumor suppressor mechanisms.

The normocytic and normochromic anemia observed in this study is referable to anemia of chronic disease in humans with cancer (Dunst et al., 2003) and in female dogs with mammary tumors (Silva et al., 2014). Almost any chronic disorder with an inflammatory component will initiate the process that causes anemia and, among them, also malignant neoplasm that causes necrosis and/or inflammation around or within the neoplasm (Stockham and Scott, 2008). The prevalence and clinical relevance of the presence of anemia in cats with mammary neoplasms is poorly reported. However, in humans with breast cancer there is a high incidence of anemia at diagnosis and correlation with poorer response to treatment, prognosis and survival (Leonard, Untch and von Koch, 2005). In this study, anemia was only observed in one animal.

The MCHC is a measure of the average hemoglobin concentration in RBC, associated with tumor progression and angiogenesis as well as tumor cell metastasis. Previous studies show that MCHC is an independent biomarker in patients with resectable lung cancer (Qu et al, 2014), and that lower preoperative MCHC value was associated with worse prognosis after hepatectomy, as it reflected unfavorable histopathological characteristics of the tumor and represented an independent prognostic factor for patients with hepatocellular carcinoma (Kong et al., 2020).

In the present study, MCHC was shown to be an independent predictive factor for OS in feline mammary cancer. Patients with low MCHC have decreased oxygen-carrying capacity in erythrocytes leading to the hypoxic microenvironment in cancer (Qu et al., 2014). Hypoxia can induce changes in the cellular genome that can promote tumor progression to enhance tumor growth and angiogenesis (Vaupel and Mayer, 2007). In the process of hypoxia-induced malignant progression, tumors may have more possibilities for local invasive growth and distant tumor cell metastasis, resulting in a poor prognosis (Osinsky, Zavelevich and Vaupel, 2009).

Platelet count was defined as an independent prognostic factor in the multivariate analysis for OS and this is the first study that observe this prognostic parameter in FMC. This probably occurred because platelet activation at the site of tissue injury contributes to the initiation of a cascade of events that promote tumorigenesis. In fact, platelets release a wide variety of proteins, including growth and angiogenic factors, lipids and extracellular vesicles rich in genetic material, which can mediate the induction of phenotypic changes in cells, such as immune, stromal and tumor cells, and promote the carcinogenesis and metastasis formation (Contursi et al., 2018) resulting in poor prognosis of cancer patients (Coffelt, Wellenstein and de Visser 2016). In addition, high platelet count is related to the development of metastasis in HBC (Leblanc et al., 2014).

ALB, as a routine item in the biochemical tests of patients, reflects nutritional levels, which are also associated with chronic inflammatory responses. Low levels of ALB can occur in patients with malnutrition and chronic infection. At the same time, patients with low levels of ALB have a worse prognosis, being considered an independent indicator of adverse prognosis (Chang et al., 2015; Gupta and Lis, 2010). In the present study, the ALB of all patients was within the reference value and was not a significant prognostic factor for OS and DFS.

Impaired kidney function, as reflected by elevated serum creatinine levels, is associated with decreased survival in cancer patients, although the exact mechanism remains unknown (Aung et al., 2014). In addition, chronic low-grade inflammation can lead to intrarenal vascular dysfunction (resulting in elevated serum creatinine levels) and transvascular loss of macromolecules (i.e., decreased ALB) (Aung et al., 2014; Jørgensen et al., 2008).

We found some limitations throughout the process of carrying out this research. Cases from the years 2012 to 2021 were analyzed, checking hospital records and manually selecting all cats undergoing mastectomy. However, due to the retrospective nature of the study, some of the data were no longer available, leading to exclusions of some patients reducing the sample size. After that, we had a total of 18 cats with all the necessary factors to participate in this retrospective study.

The only type of treatment used in these animals was surgical removal through radical, regional mastectomy or lumpectomy. The diagnostic method used consisted only of histological classification. Although the renowned importance of immunohistochemistry in refining the diagnosis, this technique is not routinely performed in the authors' institution due to the high cost and necessary equipment. In the future, the study of these hematological parameters could be compared to and associated with the evaluation of additional biomarkers in order to provide more accurate prognostic information and guide clinical decisions to improve the quality of life and survival time of feline patients with mammary carcinomas.

3.5. Conclusion

Monocytes, platelets and creatinine are easily available, minimally invasive and cost-effective parameters that can be used as prognostic factors for OS and DFS in cats with mammary carcinoma before mastectomy. Pretreatment level of MCHC and platelet count can be considered independent prognostic factors of survival. In addition, this study contributes to the knowledge about the relationship of hematological and serum markers in cats with spontaneous mammary tumors, providing the basis for further studies that elucidate the prognostic value and validate its clinical application for HBC.

Ethical statement

All procedures were performed according to the Ethics Committee for the Use of Animals (CEUA), protocol 18/2020.

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

Authors' Contributions

The work reported in the paper has been performed by the authors, unless clearly specified in the text: Conceptualization: ESS, CCR and APBB; Data curation: ESS and CCR; Formal analysis: FLV, LRAA, TBS and ESS; Investigation: ESS, CCR, COL, VRC, TBS and PTR; Methodology: ESS and APBB; Project administration: APBB; Resources: FLV, FLDO and APBB; Software: LRAA; Supervision: APBB; Validation: FLV and APBB; Visualization: ESS, CCR, COL, VRC and PTR; Writing – original draft: ESS; Writing – review & editing: ESS, CCR and APBB. All authors read, provided feedback and approves the final protocol and manuscript.

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4. CAPÌTULO 3:

PROGNOSTIC EVALUATION OF FELINE INVASIVE MAMMARY CARCINOMAS: AN EPIDEMIOLOGICAL AND CLINICOPATHOLOGICAL ANALYSIS

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Abstract

Feline mammary carcinomas (FMCs) are generally associated with an unfavorable outcome. However, overall survival (OS) may differ according to prognostic factors. As a result, we compared, through multivariate and univariate analyses, clinical and pathological data from 24 cats with mammary neoplasms, and associated it to OS and disease-free survival (DFS). Fibroepithelial hyperplasia was found in 5 cases, in contrast to 19 cases of malignant neoplasms, among which solid carcinoma was the most frequent (20.83%). Ulceration, necrosis and adherence were negative and impacting prognostic factors in this study. OS was influenced by the speed of tumor growth, type of food and previous history of mammary cancer. Histological grading did not obtain a significant result in the multivariate analysis, but grade III carcinomas had lower DFS. Mastectomy proved to be curative in cats with fibroepithelial hyperplasia and in 5.26% of early stage carcinomas, with radical mastectomy having the highest mean OS.

Keywords: female cat, histologic grade, mammary tumors, prognostic factor, survival

4.1. Introduction

Feline mammary carcinomas (FMCs) are common neoplasms and constitute approximately 17% of all neoplasms in this species (1-2). FMCs correspond to one of the most common causes of cancer-related death in cats, occurring predominantly in middle-aged animals (3) and mostly classified as malignant and aggressive (2). The pathogenesis of FMCs is related to genetic and environmental factors, however, environmental factors are believed to be the most significant (4).

The reference therapeutic approach for FMCs is surgical excision (5), with the exception of cases of inoperable neoplasms, such as inflammatory carcinomas and large nodules or in cases of distant metastasis (6-7). In some cases, adjuvant therapy is indicated, such as chemotherapy, immunotherapy, radiotherapy or a combination of these treatments (2, 8-9).

Although the FMCs tend to be biologically aggressive, OS may vary according to clinical staging after tumor excision (10-13). Due to the scarcity of knowledge about the factors involved in the development of mammary tumors and the success of their treatment, the aim of this study was to establish: (i) the most prevalent mammary tumors in cats, (ii) the relationship between their predictive and prognostic factors, and (iii) their correlations with overall survival (OS) and disease-free survival (DFS).

4.2. Materials and methods

Epidemiological and clinicopathological data of 24 feline females undergoing mastectomy were obtained from the medical records of the Veterinary Hospital of the University Federal of Viçosa (HVT – UFV), between January 2012 and May 2021. All procedures were performed according to the Ethics Committee for the Use of Animals (CEUA), protocol 18/2020.

Among the data collected were:

1) Clinical history: age at diagnosis, body weight, breed, type of food, body condition score (BCS) according to Baldwin et al. (14), reproductive status, hormone administration and history of previous problems.

2) Clinicopathological history: tumor location, number of tumors (single or multiple), tumor size, (T1: <2 cm; T2: 2–3 cm and T3: >3 cm), ulceration, adherence, tumor necrosis, consistency (firm or soft), surface (irregular or

regular), growth slow (more than 4 months) or fast (between 1 and 2 months), vascular invasion (evaluated in histopathological examination), local recurrence, regional lymph node status at diagnosis, presence of distant metastases and assessment of surgical margins (evaluated in histopathological examination).

All patients underwent complementary exams, including chest radiography (3 projections), total abdominal ultrasound and laboratory tests (blood count, liver and renal profile). The cases were classified by clinical staging for feline mammary tumors, according to the TNM system (size, lymph node involvement and distant metastases) proposed by the World Health Organization and categorized into stages: stage I ($T_1N_0M_0$ – tumors <2 cm), stage II ($T_2N_0M_0$ – tumors between 2–3 cm), stage III ($T_3N_{0-1}M_0$ – tumors >3 cm) and stage IV (T any $N_{0-1}M_1$) (8, 15-16). Finally, the animals were classified into early stage (I–III) and advanced clinical stage (IV).

The surgical procedure was classified as suggested by Fossum et al. (17). All tumors were fixed in 10% buffered formalin solution for 48 hours, dehydrated in increasing solutions of ethyl alcohol, cleared in xylene and embedded in paraffin. Histological sections (3µm) were obtained and stained with hematoxylin and eosin (H/E).

Tumors were classified according to the consensus for the diagnosis, prognosis and treatment of FMCs (18) and evaluated according to the Nottingham System classification (19). The worst prognosis lesion was selected in animals with multiple tumors.

After surgery, patients were followed up every six months for a minimum period of 12 months and tutors were contacted by telephone in cases of loss of clinical follow-up. Local progression was categorized as recurrence close to the previous resection site. DFS was defined as the period between surgery and the development of recurrences and/or distant metastases. OS was defined as the period between surgical excision of the tumor and disease-related death. Animals whose death occurred from unknown causes and with lost follow-up were not considered.

4.2.1. Statistical analyses

For data analysis the Statistical Analysis System (SAS OnDemand) was used.

Quantitative variables were analyzed by Bartlett test and Kolmogorov–Smirnov test to evaluate homogeneity of variances and normality of errors, respectively.

Frequency of qualitative variables between the two groups (neoplastic and non–neoplastic) were arranged in contingency tables and analyzed by Fisher's exact test (Freq Procedure).

The OS time and the DFS were analyzed by survival analysis (Lifetest Procedure) and the comparisons among the strata were performed by Log–Rank test. Significance level adopted was $P<0.05$.

4.3. Results

A total of 24 cases were included, non-neoplastic lesions accounted for 20.83% (5/24), while 79.17% (19/24) were classified as malignant neoplasms. Ages ranged from nine months to 16 years, with an average of 9.14±4.18 years and 3.54±0.73 kg at diagnosis, with 12.5% being overweight. Animals with malignant neoplasms had an average age of 10.32±3.1 years, while cats with non-neoplastic lesions had an average age of 1.63±1.79. 20.83% (5/24) of the cases corresponded to pure breeds, Persian (3/5) and Siamese (2/5), in contrast with 79.17% (19/24) of mixed breed. Reproductive status showed that 50% (12/24) were intact females and 50% (12/24) had already been spayed at the time of diagnosis. A significant association was observed between hormone administration and the incidence of mammary tumors ($P < 0.0411$).

The diet consisted of cat food in 70.83% (17/24) of the cases, in addition to cat food and homemade food in 12.5% (3/24) and homemade food with various snacks in 16.67% (4/24) for the animals.

Mammary neoplasm had already been diagnosed in 79.17% (19/24) of the animals included in the study. Initial clinical staging (I–III) was described in 87.5% (21/24) of cats with mammary neoplasms, and 12.5% (3/24) of cats were classified in advanced clinical staging (IV). Radical mastectomy was the most common surgical technique, being used in 62.5% (15/24) of the cases, followed by regional mastectomy which comprised 25% (6/24) and ovariohysterectomy (OVH) concomitant to the surgical procedure represented only 8.33% (2/24). Among the epidemiological and clinical staging characteristics analyzed, a significant association was only observed between the administration of hormones and the incidence of mammary tumors in 54.17% (13/24) of the female cats ($P < 0.0411$). Epidemiological information for the studied neoplasms is detailed in Table 1.

Table 1. Epidemiological characteristics and clinical staging of cats treated at HVT diagnosed with mammary gland tumors.

	Non-neoplastic Lesions (n=5)	Malignant Neoplasms (n=19)	P value
Age			
<10 years	5 (100%)	13 (68.42%)	0.5392
≥10 years	0	6 (31.58%)	
Breed			
Mixed breed	5 (100%)	14 (73.68%)	0.5440
Pure breed	0	5 (26.32%)	
Weight			
<3 kg	1 (20%)	5 (26.32%)	1.0000
≥3 kg	4 (80%)	14 (73.68%)	
BCS			
1	0	0	
2	1 (20%)	2 (10.53%)	
3	4 (80%)	14 (73.68%)	1.0000
4	0	3 (15.79%)	
5	0	0	
Type of food			
Cat food	4 (80%)	13 (68.42%)	
Cat food + homemade food	0	3 (15.79%)	1.0000
Cat food + snacks	1 (20%)	3 (15.79%)	
Reproductive status			
Spayed	1 (20%)	11 (57.89%)	0.3168
Intact	4 (80%)	8 (42.11%)	
Hormone Administration			
No	0	11 (57.89%)	0.0411
Yes	5 (100%)	8 (42.11%)	
Disease history			
Denied	4 (80%)	13 (68.42%)	1.0000
Mammary neoplasm	1 (20%)	5 (26.32%)	
Pyometra	0		
Clinical stage (TNM)			
I	0	6 (31.58%)	
II	1 (20%)	5 (26.32%)	0.2426
III	4 (80%)	5 (26.32%)	
IV	0	3 (15.78%)	
Surgical technique			
Lumpectomy	0	1 (5.26%)	
Regional mastectomy	2 (40%)	4 (21.05%)	
Radical mastectomy	3 (60%)	12 (63.16%)	0.6080
Combination of techniques	0	2 (10.53%)	
Concomitant OVH			
No	4 (80%)	18 (94.74%)	0.3804
Yes	1 (20%)	1 (5.26%)	

BCS – Body condition score: 1 – severely underweight, 2 - underweight, 3 – ideal weight, 4 - obesity e 5 – severe obesity. **OVH** – Ovariohysterectomy. **TNM** – T (tumor size), **N** (lymph node metastasis) e **M** (distant metastasis). Similar group characteristics are verified by a nonsignificant P–value (0.05.) using Fisher test on categorized variables.

Among the lesions there were: 54.17% (13/24) in more than one mammary gland, 20.83% (5/24) exclusively in the abdominal and 20.83% (5/24) in the thoracic. 37.5% (9/24) were single lesions and 62.5 % (15/24) multiple tumors. 33.33% (8/24) of the tumors were smaller than 2.0 cm and 45.83% (11/24) were larger than 3.0 cm in diameter. Ulceration was observed in 33.33% (8/24) of cats with malignant mammary neoplasms, with adherence represented 58.33% (14/24) of cases. 54.17% (13/24) of neoplasms grew rapidly while 45.83% (11/24) grew slowly. 54.17% (13/24) of tumors had firm consistency and irregular surface at diagnosis. Distant metastases occurred in 8.33% (2/24) of the cases and local recurrence was observed in 33.33% (8/24) of the cases with malignant neoplasms. Among the clinicopathological information analyzed, significant association was only observed between necrosis and malignant neoplasms (73.68%) ($P < 0.0059$) and significant difference was found between the surgical margins ($P < 0.0238$). Clinicopathological information for the neoplasms studied are detailed in Table 2.

Table 2. Clinicopathological characteristics of mammary gland neoplasms in cats treated at the HVT.

	Non-neoplastic Lesions (n=5)	Malignant Neoplasms (n=19)	P value
Adherence			
Absent	1 (20%)	9 (47.37%)	0.3577
Present	4 (80%)	10 (52.63%)	
Ulceration			
Absent	5 (100%)	11 (57.89%)	0.1304
Present	0	8 (42.11%)	
Tumor necrosis			
Absent	5 (100%)	5 (26.32%)	0.0059
Present	0	14 (73.68%)	
Consistency and surface			
Firm and irregular	2 (40%)	11 (57.89%)	
Firm and regular	2 (40%)	7 (36.85%)	0.4522
Soft and regular	1 (20%)	1 (5.26%)	
Tumor growth			
Slow	1 (20%)	10 (52.63%)	0.3271
Fast	4 (80%)	9 (47.37%)	
Tumor size			
T1	1 (20%)	7 (36.84%)	
T2	0	5 (26.32%)	0.2340
T3	4 (80%)	7 (36.84%)	
Number of tumors			
Single	3 (60%)	6 (31.58%)	0.3256
Multiple	2 (40%)	13 (68.42%)	

Location of the tumor			
Thoracic	2 (40%)	3 (15.79%)	
Abdominal	1 (20%)	4 (21.05%)	
Inguinal	0	1 (5.26%)	---
Multicenter	2 (40%)	11 (57.89%)	
Distant metastasis			
No	5 (100%)	17 (89.47%)	1.0000
Yes	0	2 (10.53%)	
Vascular invasion			
No	5 (100%)	18 (94.74%)	1.0000
Yes	0	1 (5.26%)	
Surgical margins			
Free	1 (20%)	1 (5.26%)	
Exiguous	0	12 (63.16%)	0.0238
Compromised	4 (80%)	6 (31.58%)	
Local recurrence			
No	5 (100%)	11 (57.89%)	0.1221
Yes	0	8 (42.11%)	

T1 – Tumors <2 cm. **T2 –** Tumors entre 2–3 cm. **T3 –** Tumors >3 cm. Similar group characteristics are verified by a nonsignificant P–value (0.05.) using Fisher test on categorized variables.

Fibroepithelial hyperplasia 20.83% was the most common non-neoplastic epithelial lesion. While, malignant neoplasms presented solid carcinoma 20.83% (5/24) as the most frequent histological type, followed by papillary carcinoma 16.67% (4/24) and tubular carcinoma 12.50% (3/24). Among the special types of carcinoma, micropapillary carcinoma 12.50% (3/24) was the most observed. Carcinosarcoma was the most frequently found histological type within the classification of sarcomas, present in 8.33% (2/24) of the animals (Table 3).

Table 3. Histological classification and frequency of mammary tumors diagnosed in cats treated at the HVT.

Tumor Classification	Histological Classification	N	%
1. Non-neoplastic lesion	1.1 Fibroepithelial hyperplasia	5	20.83
N = 5 (20.83%)	**2.1 Carcinomas**		
	2.1.1 Cribiform carcinoma	1	4.17
	2.1.2 Papillar carcinoma	5	20.83
	2.1.3 Tubular carcinoma	2	8.33
2. Malignant Neoplasms	2.1.4 Solid carcinoma	5	20.83
N = 19 (79.17%)	**2.2 Special type Carcinomas**		
	2.2.1 Micropapillary carcinoma	3	12.50
	2.2.2 Mucinous carcinoma	1	4.17
	2.3 Sarcomas		
	2.3.1 Carcinosarcoma	2	8.33

4.3.1. Univariate survival analysis

Among the data obtained from the 24 feline females, only 19 had malignant neoplasms, of which one was excluded from the OS analysis due to insufficient follow-up data, totalizing only 18 feline females included in the OS analysis. Mixed breed animals had the lowest average OS (297 days) and did not reach the median. Pure breed animals, on the other hand, had a higher average of OS (364 days) and reached the median ($P<0.9757$). OS did not seem to be influenced by age ($P<0.2599$) and hormone administration ($P<0.4259$), since in both situations the animals reached the median.

The history of previous problems significantly interfered with the OS. Cats with a history of mammary neoplasm had a lower average OS (115 days) and did not reach the median and animals without a history of diseases had a higher average of OS (422 days) and reached the median ($P<0.0082$; Figure 1A).

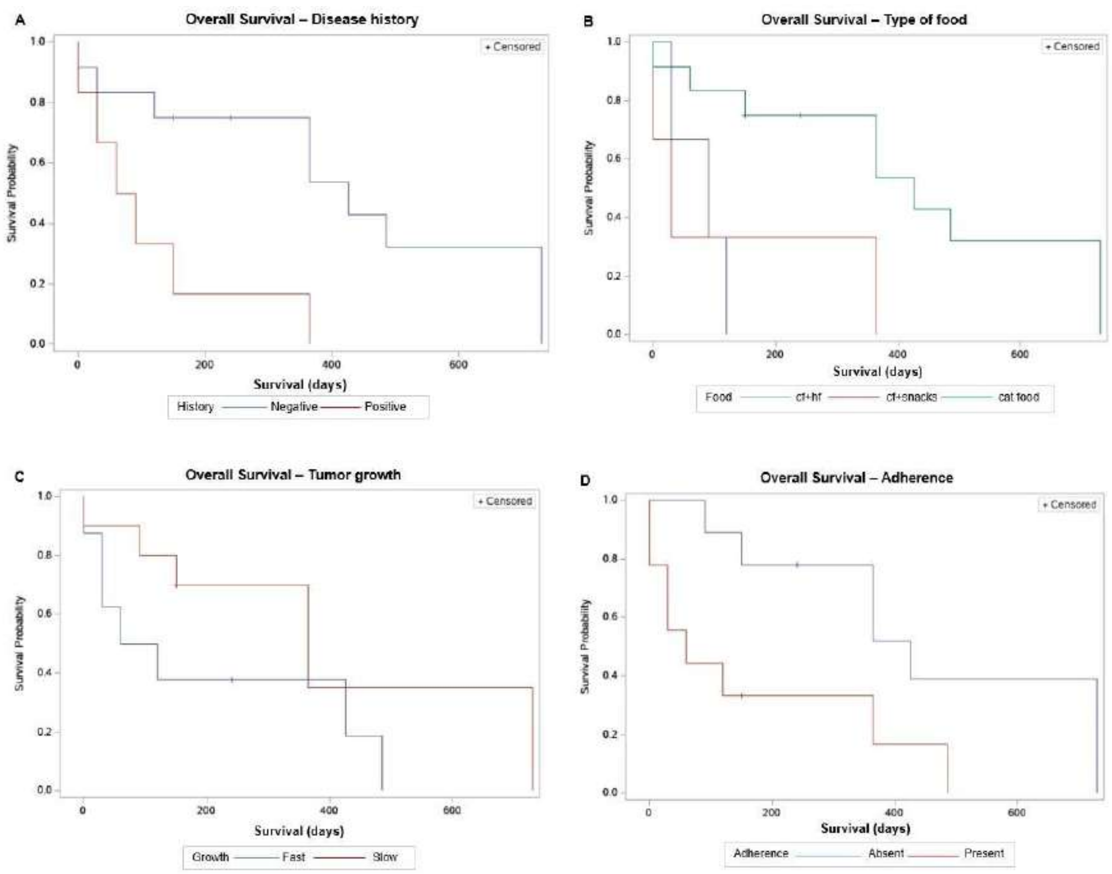
The type of food consumed by cats with malignant carcinomas significantly interfered with OS. Animals that consumed cat food in association with homemade food and cat food with snacks had the lowest average OS (80 and 131 days), respectively, and did not reach the median. On the other hand, the cats that were fed exclusively cat food had the highest average of OS (427 days) and reached the median ($P<0.0163$; Figure 1B).

In cats diagnosed with malignant neoplasms, no significant association was observed between tumor size and OS ($P<0.9757$). Groups T1 and T2 reached the median. However, the T3 group did not reach the median and had a lower average OS (298 days). Fast tumor growth had a lower average OS (200 days) and did not reach the median, while slow tumor growth had a higher average OS (407 days) and reached the median ($P<0.1128$; Figure 1C). A significant association was found between tumor adherence and OS. Cats with adhered tumors had a lower average OS (168 days) and did not reach the median when compared with non-adhered tumors that had a higher OS (460 days) and reached the median ($P<0.0219$; Figure 1D).

Despite not showing a significant association, cats in the initial clinical stage (I–III) had higher OS ($P<0.9875$) than those diagnosed in the advanced clinical stage (IV) with an average OS of 283 days. Cats with distant metastasis had a lower averge OS (212 days) and did not reach the median, while those without metastasis at the time of diagnosis had a higher OS (332 days) and reached the median ($P<0.4469$; Figure 1E).

The type of procedure performed interfered with the OS, cats that underwent radical mastectomy had a higher OS (350 days) and reached the median. However, other types of procedures performed, such as lumpectomy and regional mastectomy, had a lower average of OS (231 days) and did not reach the median (P<0.4147).

Cats diagnosed with papillary carcinoma had a higher average of OS (328 days), while micropapillary carcinomas had an average OS of 263 days and both reached the median. On the other hand, solid carcinomas had a lower average of OS (188 days) and did not reach the median (P<0.7503; Figure 1F). In addition, of the histological grading performed in 19 cases of malignant neoplasms, 5.26% (1/19) were classified as grade I, 10.53% (2/19) as grade II and 84.21% (16/19) as grade III. Overall, cats with neoplasms of histological grades II and III had an average OS of 365 and 307 days, respectively, and reached the median (P<0.7464).



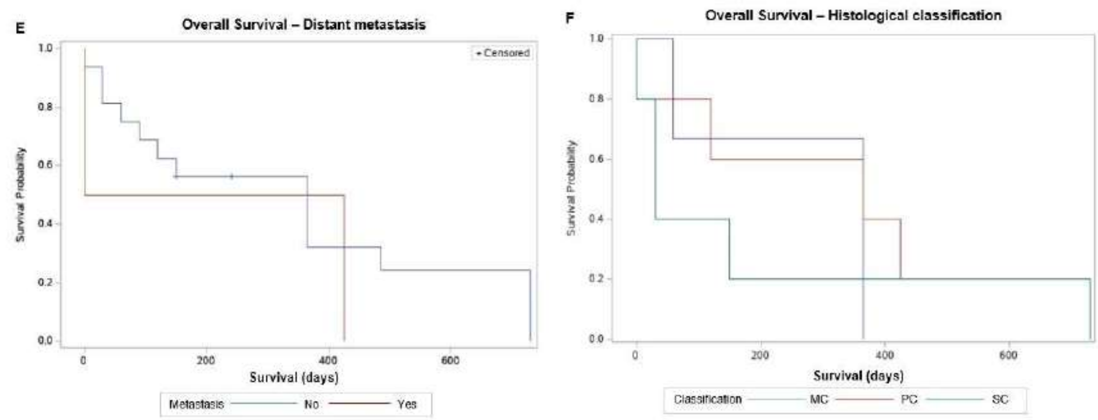


Figure 1. Kaplan–Meier overall survival curve of cats with malignant mammary neoplasms. Malignant mammary neoplasms classified according to: **A** – History of mammary neoplasm (n=6) and no history (n=12). **B** – Type of food: cat food and homemade food (n= 3), cat food and snacks (n= 3) and cat food only (n= 12). **C** – Tumor growth speed: slow (n= 11) and fast (n= 8) growth. **D** – Adherence present (n=9) and absent (n=9). **E** – Presence of metastasis: no (n= 16) and yes (n= 2). F – Histological type. PC: papillary carcinoma (n=5), SC: solid carcinoma (n=5) and MC: micropapillary carcinoma (n=3) median of 365, 365 and 30 days, respectively.

Tumors with necrosis in the histopathological diagnosis had a lower average of OS (244 days) and did not reach the median. Meanwhile, tumors without necrosis had a higher OS (516 days) and reached the median (P<0.0879). Ulcerated tumors had a lower average of OS (232 days), while non-ulcerated tumors had a higher OS (367 days) and both reached the median (P<0.2534). The free and narrow surgical margins had higher averages of OS (730 and 326 days), respectively. The compromised margins had a lower OS (217 days) and did not reach the median (P<0.3096).

From the data available for DFS in 18 cases, we observed significant differences between DFS and type of food and disease history (P<0.0005, P<0.0009, Figure 2A e B, respectively, Table 4).

Cats with solid carcinomas had a tendency to be shorter DFS, although not shown by a significant difference, while those with papillary carcinomas had a longer DFS (P<0.5830; Table 5). DFS was also lower in cats with grade III carcinomas (P<0.6689). DFS was not influenced by ulceration, adherence and rapid growth in the clinical presentation (Table 5).

Table 4. One-year corrected survival rates in cases with available follow-up data with epidemiologic characteristics and clinical stage[a]

		Overall survival (*n*=18)			**Disease-free survival** (*n*=18)		
		n	Median survival (days)	Average 1-year survival rate *n* (%)	*n*	Median survival (days)	Average 1-year survival rate *n* (%)
Age	<10	13	425	4 (30.77)	13	365	4 (30.77)
	>10	5	365	1 (20)	5	120	1 (20)
	P		0.2599			0.1801	
Breed	Mixed breed	13	150	3 (23.08)	13	90	1 (7.69)
	Pure breed	5	425	2 (40)	5	365	3 (60)
	P		0.7692			0.6369	
Weight	<3kg	5	485	1 (20)	5	485	2 (40)
	>3kg	13	150	2 (15.38)	13	90	3 (23.08)
	P		0.3291			0.3334	
BCS	1	0	0	0	0	0	0
	2	2	455	1 (50)	2	425	1 (50)
	3	13	365	3 (23.08)	13	90	3 (23.08)
	4	3	150	0	3	120	0
	5	0	0	0	0	0	0
	P		0.8806			0.9487	
Type of food	Cat food	12	425	5 (41.67)	12	365	4 (33.33)
	Cat food + homemade food	3	90	0	3	90	0
	Cat food + snack	3	30	0	3	0	0
	P		0.0163			0.0005	
Reproductive status	Intact	8	365	3 (37.5)	8	365	2 (25)
	Spayed	10	257	2 (20)	10	105	2 (20)
	P		0.9305			0.9686	
Hormone administration	No	11	365	3 (27.27)	11	365	2 (18.18)
	Yes	7	365	2 (28.57)	7	120	1 (14.29)
	P		0.4259			0.5630	
Disease history	Negative	12	425	5 (41.67)	12	365	4 (33.33)
	Positive	6	75	0	6	45	0
	P		0.0082			0.0009	
Clinical Stage (TNM)	I	6	257	1 (16.67)	6	105	1 (16.67)
	II	5	365	2 (40)	5	365	2 (40)
	III	4	380	0	4	380	0
	IV	3	425	0	3	365	0
	P		0.9875			0.9589	
Surgical technique	Radical mastectomy	13	365	4 (30.77)	13	365	3 (23.08)
	Other techniques	5	60	1 (20)	5	60	1 (20)
	P		0.4147			0.5281	

[a]Survival values are percentages for cumulative survival determined by Log-Rank test. Significance level adopted was <0.05.

Table 5. One-year corrected survival rates in cases with available follow-up data with clinicopathological characteristics[a]

		Overall survival (*n*=18)			**Disease-free survival** (*n*=18)		
		n	Median survival (days)	Average 1-year survival rate *n* (%)	*n*	Median survival (days)	Average 1-year survival rate *n* (%)
Adherence	Absent	9	425	4 (44.44)	9	365	3 (33.33)
	Present	9	60	1 (11.11)	9	60	1 (11.11)
	P		0.0219			0.0621	
Ulceration	Absent	11	365	4 (36.36)	11	365	4 (36.36)
	Present	7	365	1 (14.29)	7	60	0
	P		0.2534			0.1389	
Tumor necrosis	Absent	5	730	2 (40)	5	730	2 (40)

	Present	13	120	3 (23.08)	13	90	2 (15.38)
	P		0.0879			0.0719	
Consistency and surface	Firm and irregular	10	365	3 (30)	10	227	2 (20)
	Firm and regular	7	365	2 (28.57)	7	365	2 (28.57)
	Soft and regular	1	30	0	1	30	0
	P		0.2426			0.3345	
Tumor growth	Slow	10	365	3 (30)	10	365	3 (30)
	Fast	8	90	1 (12.5)	8	90	1 (12.5)
	P		0.1128			0.1767	
Tumor size	T1 (<2cm)	7	365	1 (14.29)	7	120	1 (14.29)
	T2 (2-3cm)	5	365	2 (40)	5	365	2 (40)
	T3 (>3cm)	6	227	1 (16.67)	6	197	1 (16.67)
	P		0.9757			0.9843	
Number of tumors	Single	6	135	1 (16.67)	6	105	1 (16.67)
	Multiple	12	365	3 (25)	12	365	2 (16.67)
	P		0.6236			0.6932	
Histological type (*n*=13)	Micropapillary carcinoma	3	365	1 (33.33)	3	60	0
	Papillary carcinoma	5	365	2 (40)	5	365	5 (100)
	Solid carcinoma	5	30	0	5	30	0
	P		0.7503			0.5830	
Histological grade	Grade I	1	365	1 (100)	1	365	1 (100)
	Grade II	2	365	0	2	365	0
	Grade III	15	365	4 (26.67)	15	120	3 (20)
	P		0.7464			0.6689	
Distant metastasis	No	16	365	4 (25)	16	242	4 (25)
	Yes	2	212	0	2	182	0
	P		0.4469			0.4279	
Surgical margin	Free	1	730	1 (100)	1	730	1 (100)
	Exiguous	11	365	3 (27.27)	11	365	2 (18.18)
	Compromised	6	90	1 (16.67)	6	60	1 (16.67)
	P		0.3096			0.3452	

[a]Survival values are percentages for cumulative survival determined by Log-Rank test. Significance level adopted was <0.05.

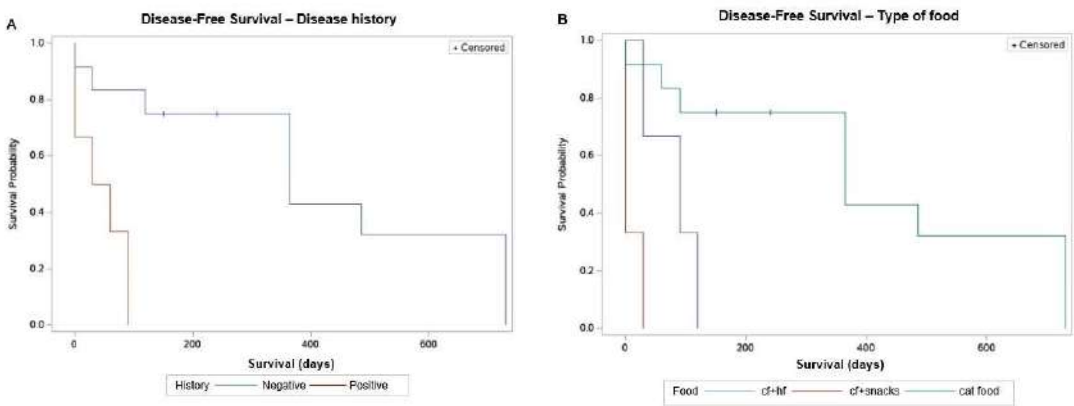


Figure 2. Kaplan–Meier disease-free survival curve of cats with malignant mammary neoplasms Malignant mammary neoplasms classified according to: **A –** History of previous mammary neoplasm (n= 6) and no history (n= 12). **B –** Type of food: cat food and homemade food (n= 3), cat food and snacks (n= 3) e only cat food (n= 12).

4.4. Discussion

FMCs are highly malignant and often associated with a poor prognosis, making them a major cause of cancer-related death in cats. Thus, the study of epidemiological, clinicopathological, histological and follow-up characteristics aims to provide valuable information about the highly aggressive behavior of this neoplasm, predictive and prognostic factors, disease-free time and overall survival time. It is worth noting that such prognostic factors can help predict outcomes and guide decision making.

Epidemiological studies reveal the existence of breed predisposition for the development of mammary neoplasms in felines, with most studies indicating the Siamese breed as the most affected (20-21). This result is different from that found by Togni et al. (22), and as in the present study, we observed a greater involvement of mixed breed animals and less of Persians followed by Siamese, which may have occurred due to miscegenation and the proportion between the different breeds in the analyzed cat population.

The average age at diagnosis was 10 years for cats with malignant carcinomas, corroborating with Núñez and Bouda (4), Togni et al. (22) and Peleterio (23) and fibroepithelial hyperplasia occurred in young animals as observed by Peleteiro (23). Age at diagnosis did not affect OS time, but reduced DFS. Mixed feeding can cause an imbalance in the diet and, thus, increase the chances of the animal developing neoplastic masses (24). Even when provided a balanced diet with commercial cat food, the addition of other foods increases caloric levels, predisposing to obesity and neoplasms (25), therefore, as observed in this study, feeding animals exclusively with commercial cat food results in a better average OS. This study also shows that mixed feeding significantly interfered in OS and DFS.

Spayed animals are less likely to develop tumors (7) and that the administration of contraceptives for estrus inhibition is associated with a higher risk for the development of FMCs and fibroepithelial hyperplasia (26-28). In cats, OVH performed before one year shows a significant reduction in the risk of developing a mammary carcinoma (26), with no benefits being observed in the prevention of FMCs when performed after 24 months of age (29). However, in our study, only half of the cats with malignant neoplasms had undergone OVH and almost half of the owner reported regular administration of exogenous hormones. All animals diagnosed with fibroepithelial hyperplasia received administration of exogenous hormones.

Tumor size is a commonly used diagnostic indicator due to its correlation with prognosis in FMCs (30), however, in our study we observed only a weak correlation between tumor diameter and OS for tumors larger than 3 cm in diameter. Despite this, our findings are consistent with reports that indicate that T3 tumors are associated with lower OS (31-32). Tumor growth speed is a variable that has not yet been explored by FMC studies. However, we can observe that rapid tumor growth interferes considerably in OS and DFS, reducing them by 1/3 when compared to slow tumor growth.

In our study, the thoracic and abdominal mammary glands were more affected, corroborating with some studies (22, 33) and differing from others (34-35). In addition, among the cases analyzed, several had both chains affected, thus demonstrating that the existence of multiple mammary tumors is relatively common in the feline species (6).

According to Wypij et al. (36), benign mammary tumors appear in felines in a much lower proportion than malignant ones, however, in our study we did not observe benign neoplasms, probably due to the size of the analyzed sample. FMCs are very aggressive malignant neoplasms (13, 37), due to this aggressiveness we probably observe recurrence in a more invasive way and with a high degree of malignancy (grade III) in animals diagnosed with mammary carcinomas. The history of involvement by mammary cancer had a negative influence on OS and DFS. Furthermore, in our study, only 5.26% of the lesions were classified as grade I, similar to what was found by Seixas et al. (38) and Dagher et al. (39), and although graduation in some studies is associated with OS and DFS (38), we only observed a reduction in DFS as a function of graduation. Nonetheless, most of the tumors analyzed in our study were classified as stage III, as observed by Borrego, Cartagena and Enge (I8) and De Campos et al. (33). Furthermore, in our study, two cats with metastatic stage IV underwent palliative surgery with OS of 212 days and lower OS and DFS, a value much lower than that observed by Petrucci et al. (40) for cats in metastatic stage.

Considering the histological classification, in our study the cats with micropapillary and solid carcinoma had the worst OS, corroborating Seixas et al. (38), Mills et al. (32) and Granados–Soler et al. (41) and DFS (38). In addition, despite the sample size, some histological types found, such as mucinous carcinoma and carcinosarcoma, are described as less frequent patterns (2, 6, 33) and the development of local recurrence was not a predictive factor of OS in this study, contradicting the findings of Wood et al. (42).

No previous work has shown adherence to be a prognostic factor for due to the scarcity of data for the feline species. Despite this, our study demonstrates a favorable opinion regarding its use as a prognostic factor, since we observed it in 58.33% of the cases and it significantly reduced the OS of cats with mammary carcinoma. Meanwhile, the occurrence of ulceration and necrosis are considered prognostic factors (43) and were found in our study mainly in neoplasms with a more aggressive behavior and resulted in shorter OS and DFS times.

The evaluation of surgical margins is a parameter that has been rarely addressed in studies of FMCs and poorly correlated with OS and DFS. However, in our study, we observed that compromised surgical margins interfere with OS and DFS, reducing them considerably when comparing free and narrow margins, corroborating with few existing studies, in which surgical excision was significantly associated with a higher rate of tumor progression and lower OS (5, 42, 44).

Finally, the most eligible treatment for mammary cancer is mastectomy (5) and can be performed with or without the association chemotherapeutic agents (45). Our results showed that surgery was curative for cats with fibroepithelial hyperplasia and in 5.26% of cases with early stage carcinomas (I–III). The type of procedure performed did not demonstrate an independent prognostic value. However, the method used (radical or conservative surgery) seems to alter OS and DFS.

Together our results clearly show that history of previous mammary cancer, type of food (homemade or snack) decrease OS and DFS, whit adherence also decreasing OS. Other important factors that can decrease OS are breed, tumor size (T3), presence of metastasis, type of procedure performed (lumpectomy and regional mastectomy), presence of necrosis and tumor adherence, and compromised surgical margins. Thus, early detection and effective and targeted therapeutic options are important to prevent metastases, and improve patients' OS and DFS.

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Declaration of Conflicting Interests

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5. CAPÌTULO 4:

OVARIOHYTERECTOMY AS AN ADJUVANT THERAPY IN DOGS WITH MAMMARY CARCINOMAS

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Abstract

Mammary tumors are the neoplasms that most affect intact female dogs. Surgery is the standard treatment and has good prognosis for non-metastatic, low-grade tumors. Ovariohysterectomy (OVH) at an early age decreases the risk of developing those tumors. However, the positive effect of performing OVH at the time of tumor removal in female dogs with mammary carcinomas is not well understood. We aimed to evaluate the effect of OVH based on clinicopathological parameters and analysis of disease-free interval (DFI), cancer-specific survival (CSS) and overall survival (OS) of female dogs with mammary carcinomas. 102 dogs were included: 44 that underwent spaying concurrently with mastectomy (OVH group) and 58 that had only the tumors removed, (Non-OVH) group. For each of the explanatory variables, both groups were compared using non-significant chi-square test with P-value as an indicator of similar group characteristics. The Kaplan-Meier method was used to calculate the estimates of DFI, CSS and OS with treatment differences assessed through Log-Rank test. OVH status and the other variables were assessed by univariate Cox proportional hazards models for their effect on analyzed factors. Risk ratios were estimated with 95% confidence intervals (CI). The variables of age (> 10 years), histological grade (3) and Non-OVH group are independent prognostic factors and significantly interfere in CSS, DFI, and OS time of patients. Thus, spaying at the time of a mastectomy should always be considered in intact female dogs with mammary tumors, as it has a positive correlation with greater CSS, DFI and OS in most cases.

Keywords: female dog, mammary tumor, mastectomy, prognostic factor, survival

5.1. Introduction

Among women, breast cancer is the most common type of cancer, being the second most frequent cancer in the world and the fifth most common cause of cancer-related death (1, 2). In the canine species, mammary tumors are the most frequent type of tumor found in intact female dogs (3, 4). Surgery is the standard treatment with a good prognosis for animals with non-metastatic and low-grade tumors (5).

Canine mammary tumors (CMTs) are hormonally controlled (6). Sex steroids stimulate the growth of normal breast tissue under physiological conditions. Its proliferative effect on the epithelium can create conditions for neoplastic proliferation (5, 7, 8). Possible mechanisms involved in CMTs are progesterone-induced, which include an upregulation of growth hormone production within the mammary gland (9), leading to stem cell proliferation that could have a primary role in carcinogenesis (3, 9, 10). This occurs with each estrous cycle and makes the dog increasingly susceptible to further carcinogenesis (11, 12, 13).

The biological and molecular consequences of estrogen binding to nuclear receptors on mammary epithelial cells provide explanations for the mammary carcinogenic effects of estrogen. Upon entering cells and binding to the nuclear receptor, estrogen initiates a sequence of molecular events resulting in transcriptional alterations of estrogen-responsive genes and an increased expression of positive proliferation regulators and downregulation of anti-proliferative and pro-apoptotic genes (14, 15). This causes increased cell division, facilitated continued growth and promoted additional spontaneous mutations (14, 16, 17).

Ovariohysterectomy (OVH) at an early age significantly decreases the risk of dogs developing mammary gland tumors (3). Studies have evaluated DFS and the difference between the incidence of benign and malignant tumors in dogs diagnosed with mammary tumors depending on sterilization status (18, 19, 20), suggesting that the progression of these tumors is independent of continuous estrogen stimulation.

In this context, this study aims to evaluate the effect of OVH concomitantly with the surgical removal of tumors based on clinicopathological parameters and on the analysis of disease-free interval (DFI), cancer-specific survival (CSS) and overall survival (OS) of female dogs with mammary carcinomas.

5.2. Materials and methods

5.2.1. Data collection

The retrospective study was carried out with data from female dogs with mammary tumors and submitted to the surgical procedure of mastectomy obtained by consulting the database of the Veterinary Hospital of the Universidade Federal de Viçosa, MG, Brasil from January 2012 to December 2020. The study was designed to enroll intact female dogs with histologically confirmed mammary carcinomas without distant metastases, other serious diseases, or any previous history of mammary malignancy. The histopathological examination was carried out by the Pathology Laboratory of the Veterinary Department of the Universidade Federal de Viçosa – UFV. Each case was accounted for only once, regardless of the number of hospital visits, and the records were retrospectively evaluated. All procedures were approved by the Ethics Committee for the Use of Animals of the University under protocol 18/2020.

Age, breed, body weight, pseudo-pregnancies, clinical characteristics of the tumor (number and size), presence of ulceration, evaluation of regional lymph nodes, classification according to the TNM system (21, 22) and the type of surgical procedure performed and the histological diagnosis were retrieved from the medical records.

The database also contained standard preoperative exams, such as a complete physical examination and clinical staging, consisting of a complete blood count, serum biochemical profile, chest radiographs in 3 projections and, in some cases, abdominal ultrasounds and cytological examinations, performed at the time of diagnosis of mammary cancer. All these preliminary exams led to the decision to perform surgery.

The dogs were assigned to undergo tumor removal with concomitant OVH (OVH group) or only tumor removal (Non-OVH group). It is important to highlight that the animal's castration procedure was recommended by the veterinarian responsible for the case, however, its completion depended on authorization from the animal's owner. Surgery was performed as suggested by Fossum et al. (23). The size, location and number of tumors and the clinical status of the regional lymph nodes determined the extent of resection. Dogs with stage IV disease had the affected lymph node resected en bloc along with the mammary tumor whenever possible. OVH was performed before mastectomy in order to prevent tumor cells from seeding into the abdominal cavity (23).

Tumors were classified according to the consensus for the diagnosis, prognosis and treatment of CMTs (24, 25, 26). In addition, tumors were evaluated according to the Nottingham System classification (27). The lesion with the worst prognosis was selected in animals with multiple tumors.

After surgery, patients were followed up every six months for a minimum period of 12 months at the Veterinary Hospital and owners were contacted by phone in cases of loss of clinical follow-up. Local progression was categorized as recurrence close to the previous resection site. DFI was defined as the time interval from surgery to detection of of new tumors and/or distant metastases. CSS was defined as the period of time since surgical excision of the tumor and disease-related death. OS was defined as the period from surgical removal of the tumor until the patient's death from any cause.

The cause of death or euthanasia was recorded and classified as non-mammary (when not related to the tumor) and mammary (when related to the breast tumor).

5.2.2. Data analysis

For each of the explanatory variables, the OVH group was compared with the non-OVH group using nonsignificant chi square test results with the P-value as an indicator of similar group characteristics. Dogs lost to follow-up or still alive without disease progression were censored at the date of last known status. The Kaplan–Meier method was used to calculate estimates of overall survival (OS) time and the disease-free interval (DFI) with treatment differences assessed by the use of Log-Rank test. The OVH status and the other investigated variables were evaluated by univariable Cox proportional hazard models for their effect on DFI and OS. Time at risk was defined as days from the date of surgery to the event of interest or censoring.

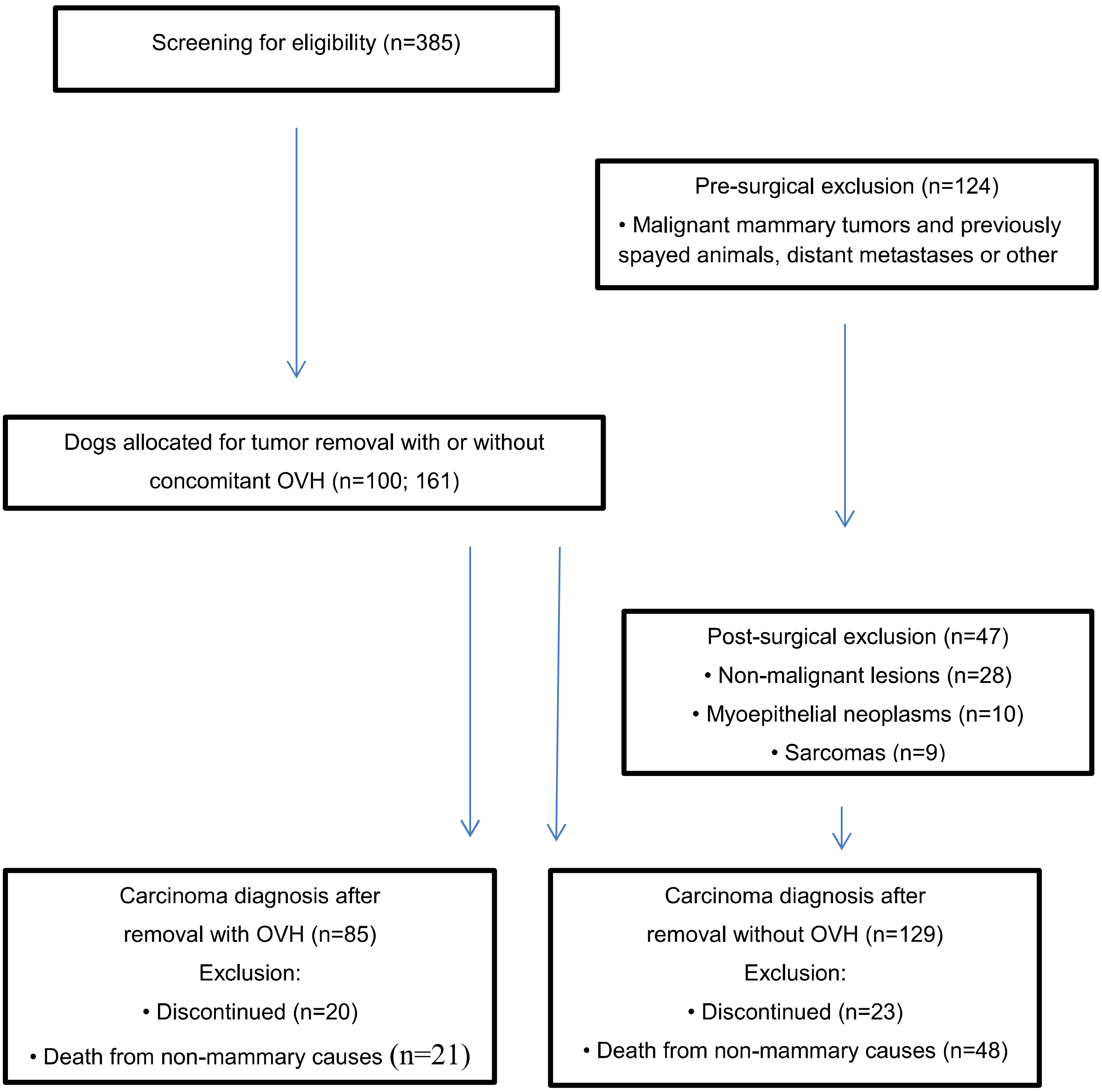
Cox's proportional hazard analysis was performed to determine the way in which OS and DFI were affected by the other co-variables (Phreg Procedure).

The hazard ratios were estimated with 95% confidence intervals (CI). Significance level adopted was 0.05.

Explanatory variables were used to predict the probability of survival for at least 1 year by multivariate logistic regression (Logistic Procedure), stepwise selection was used and only variables that were significant at P < 0.10 were kept in final model. For data analyses the Statistical Analysis System (SAS OnDemand) was used.

5.3. Results

102 of the 385 dogs evaluated for eligibility were included, with 44 dogs allocated to the OVH group and 58 to the Non-OVH group. Figure 1 depicts the application process.



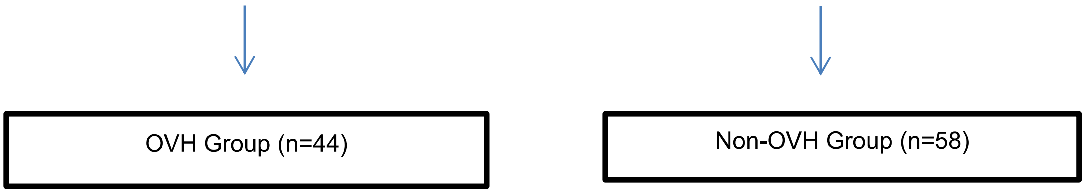


Figure 1. Enrollment flowchart in the investigation of the effect of ovariohysterectomy (OVH) at the time of tumor removal in 102 female dogs with mammary carcinomas.

Average age and weight were 8.84±2.76 years and 11.79±9.75 kg, respectively. Purebred animals represented 73.53% (75/102) of the cases, while 26.47% (27/102) were considered mixed breed. The different breeds frequency is reported in Supplementary Material (S1). 15.69% (16/102) had a history of pseudocyesis. Among animals with incidence of mammary tumors, 21.57% (22/102) received hormone administration.

The initial clinical stage was described in 92.16% (94/102) of the female dogs with mammary neoplasms and the advanced clinical stage in 7.84% (8/102), with a significant association observed between the TNM of the OVH group and the Non-OVH group (P=0.0019). Tumor removal was performed with different approaches, most frequently with regional mastectomy employed in 50% (51/102) of cases and radical mastectomy in 33.33% (34/102).

Tumor size correlated between the OVH group and the Non-OVH group, with the majority of tumors 55.88% (57/102) being smaller than 3 cm in diameter (P=0.0004). Single lesions accounted for 29.41% (30/102) and multiple lesions accounted for 70.59% (72/102) of cases. Table 1 provides an overview of all clinicopathological characteristics of the dogs and studied tumors classifyed by treatment arm.

Mixed tumor carcinoma, papillary carcinoma, tubular carcinoma and solid carcinoma were the most frequent histological types, present in 59.8% (61/102), 11.76% (12/102), 10.78% (11/102) and 9.8% (10/102) of the cases, respectively (Table 2). The histological grading resulted in: 41.18% (42/102) classified as grade I, 42.16% (43/102) as grade II and 16.67% (17/102) as grade III (Table 2).

Table 1. Description of the characteristics of 102 female dogs with mammary carcinomas allocated for hysterectomy (OVH group) or to remain intact (Non-OVH group) during tumor removal.

	Grupo OVH (n=44)	Grupo Não-OVH (n=58)	P
Age			0.1592
≤10 years	36 (81.82%)	41 (70.69%)	
>10 years	7 (15.91%)	17 (29.31%)	
Undefined	1 (2.27%)	0	
Breed			---
Mixed breed	16 (36.36%)	11 (18.97%)	
Purebred	28 (63.64%)	47 (81.03%)	
Weight			0.6886
≤10 kg	27 (61.36%)	33 (56.90%)	
>10 kg	17 (38.64%)	25 (43.10%)	
Pseudocyesis			1.0000
Absent	37 (84.09%)	49 (84.48%)	
Present	7 (15.91%)	9 (15.52%)	
Hormone administration			0.4773
No	33 (75%)	47 (81.03%)	
Yes	11 (25%)	11 (18.97%)	
Clinical Stage (TNM)			0.0019
I	34 (77.27%)	23 (39.66%)	
II	3 (6.82%)	14 (24.14%)	
III	5 (11.36%)	15 (25.86%)	
IV	2 (4.55%)	6 (10.34%)	
Surgical technique			1.0000
Lumpectomy	2 (4.55%)	3 (5.17%)	
Regional mastectomy	22 (50%)	29 (50%)	
Radical mastectomy	15 (34.09%)	19 (32.76%)	
Combination of techniques	5 (11.36%)	7 (12.07%)	
Tumor size			0.0004
T1	36 (81.82%)	25 (43.10%)	
T2	3 (6.82%)	14 (24.14%)	
T3	5 (11.36%)	19 (32.76%)	
Number of tumors			0.5111
Single	11 (25%)	19 (32.76%)	
Multiple	33 (75%)	39 (67.24%)	

T1 – tumors < 3 cm. **T2 –** tumors between 3 – 5 cm. **T3 –** tumors > 5 cm. Similar group characteristics are verified by a nonsignificant $P<0.05$ using chi square test on categorized variables.

Table 2. Distribution of female dogs based on histological type and grading of mammary carcinomas in the investigation of the effects of ovariohysterectomy (OVH group) or remaining intact (Non-OVH group) during tumor removal.

	OVH group (n=44)	Non-OVH group (n=58)	P
Histological grade			0.0550
I	24 (54.55%)	18 (31.03%)	
II	15 (34.09%)	28 (48.28%)	
III	5 (11.36%)	12 (20.69%)	
Histological type			0.7982
Mixed tumor carcinoma	26 (59.09%)	35 (60.34%)	
In situ carcinoma	0	1 (1.72%)	
Pleomorphic lobular carcinoma	1 (2.27%)	1 (1.72%)	
Micropapillary carcinoma	1 (2.27%)	3 (5.17%)	
Mucinous carcinoma	1 (2.27%)	0	
Papillar carcinoma	7 (15.91%)	5 (8.62%)	
Solid carcinoma	3 (6.82%)	7 (12.07%)	
Tubular carcinoma	5 (11.36%)	6 (10.34%)	

Similar group characteristics are verified by a nonsignificant P<0.05 using chi square test on categorized variables.

5.3.1. Follow-up and Clinical Outcome

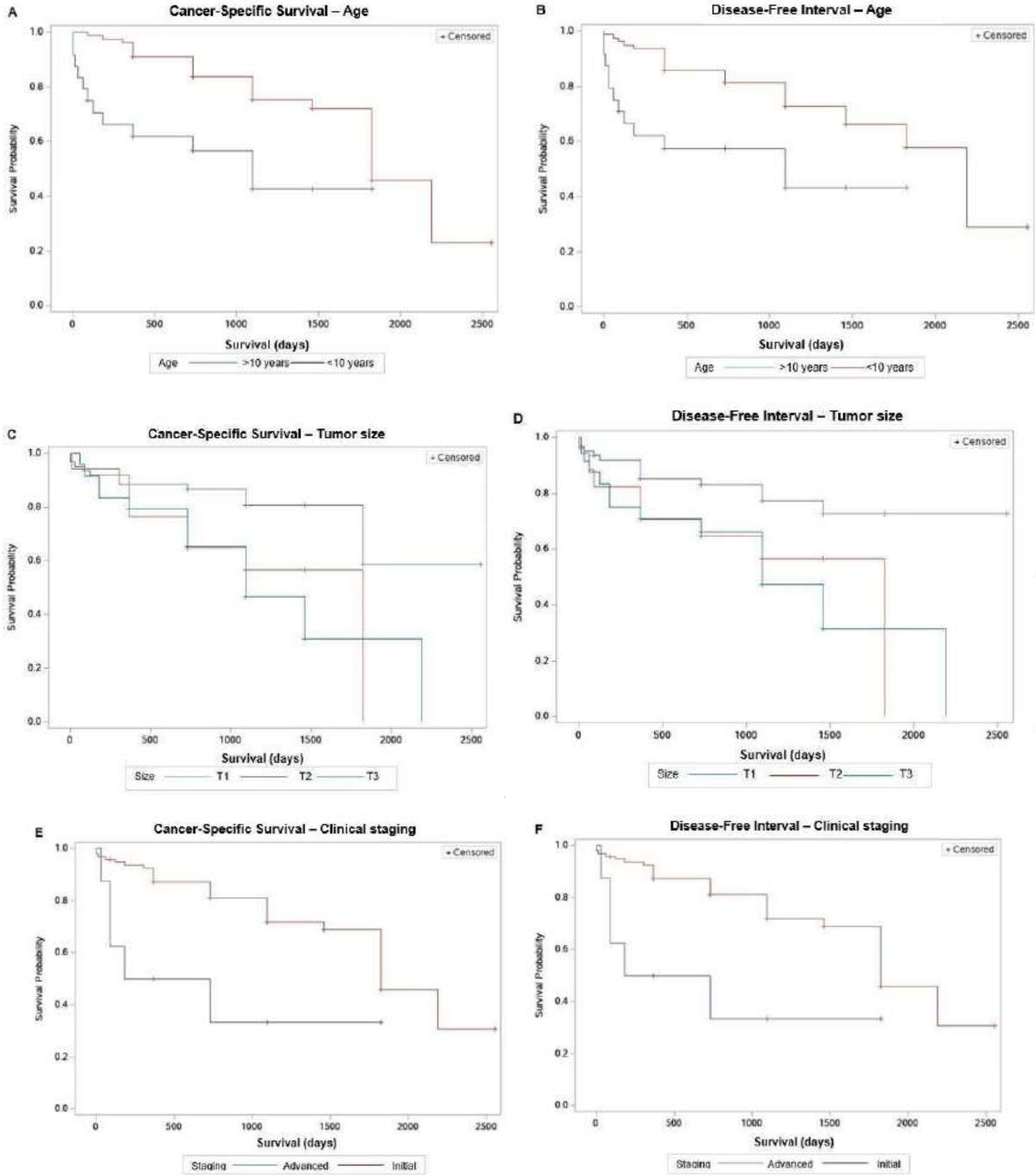
All 102 female dogs were available for regular follow-up until death or censorship with a median follow-up time of 36 months for the OVH group and 18.7 months for the Non-OVH group. None of the animals received any other treatment during the study period. Recurrence occurred in 27.27% (12/44) in the OVH group and 36.21% (21/58) in the Non-OVH group.

5.3.2. Univariate survival analysis

5.3.2.1. Cancer-specifc survival (CSS) and Disease-free interval (DFI)

A significant association was observed between age, CSS and DFI. Younger animals had higher means of CSS and DFI (1700 and 1674 days) and the oldest smaller averages of CSS and DFI (694 and 668 days) (P=0.0018; P=0.0046; Figures 2A and B). A significant association was also observed between tumor size and CSS (P=0.0064; Figure 2C) and DFI (P=0.0133; Figure 2D), the smaller the size of the tumor, the higher the CSS and DFI. Female dogs in the initial clinical stage (I-III) presented CSS (mean 1,639 days) (P=0.0007; Figure 2E) and DFI (mean 1616 days; P=0.0016; Figure 2F) significantly longer than those diagnosed in advanced clinical stage (IV) (mean 413 and 402 days, respectively).

Histological classification of tumors significantly influenced the CSS (P=0.0027; Figure 2G) and DFI (P=0.0055, Figure 2H), with mixed tumor carcinoma showing the highest means of CSS and DFI (1528 and 1460 days). Histological grades I and II had significantly higher means of CSS and DFI (P=0.0004; P=0.0010; Figures 2I and 1J), grade III had a lower average of CSS and DFI (1023 and 986 days, respectively).



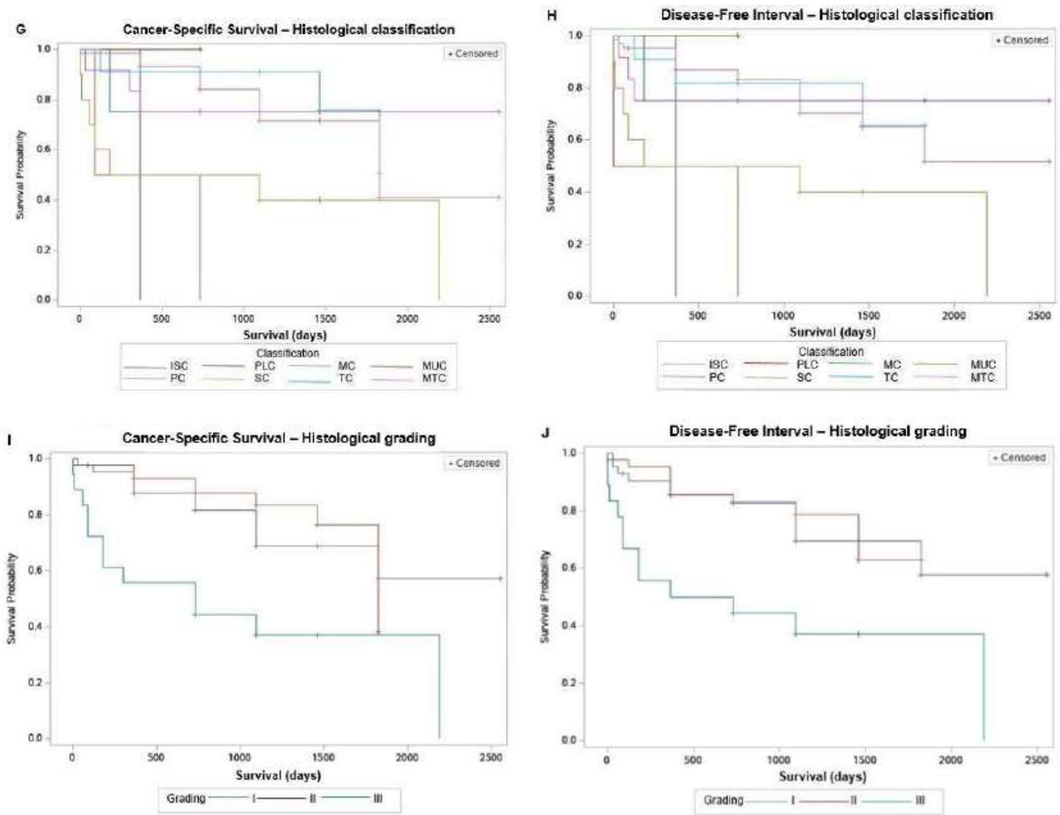


Figure 2. Kaplan–Meier cancer-specific survival and disease-free interval curve of female dogs with mammary carcinomas investigating the prognostic effect of ovariohysterectomy (OVH) performed at the time of tumor removal. Mammary carcinomas classified according to: A – CSS age: ≤10 years (1700 days) and >10 years (694 days). B –DFI age: ≤10 years (1674 days) and >10 years (668 days). C – CSS tumor size: T1 (1566 days), T2 (1268 days) and T3 (1248 days). D – DFI tumor size: T1 (1234 days), T2 (1217 days) and T3 (1,199 days). E – CSS clinical stage: I (1632 days), II (1268 days), III (1294 days), and IV (413 days). F – DFI clinical stage: I (1282 days), II (1217 days), III (1234 days), and IV (402 days). G –CSS Histological classification: ISC - *in situ* carcinoma (365 days), PLC - pleomorphic lobular carcinoma (410 days), MC - micropapillary carcinoma (180 days), MUC - mucinous carcinoma (0 days), PC - papillar carcinoma (331 days), SC - solid carcinoma (1019 days), TC - tubular carcinoma (1614 days), MTC - mixed tumor carcinoma (1528 days). H - DFI Histological classification: ISC - *in situ* carcinoma (365 days), PLC - pleomorphic lobular carcinoma (365 days), MC - micropapillary carcinoma (180 days), MUC - mucinous carcinoma (0 days), PC - papillar carcinoma (110 days), SC - solid carcinoma (1019 days), TC - tubular carcinoma (1238 days), MTC - mixed tumor carcinoma (1460 days). I – CSS histological grade: I (1470 days), II (1600 days) and III (1023 days), respectively. J – DFI histological grade: I (1454 days), II (1254 days) and III (986 days), respectively.

CSS and DFI were not influenced by weight, hormone administration, pseudocyesis, number of tumors and type of surgical procedure performed. CSS and DFI information for mammary carcinomas in investigating the effect of ovariohysterectomy (OVH group) or to remain intact (Non-OVH group) during tumor removal are detailed in Table 3 (data from both groups were analyzed together).

Table 3. One-year corrected survival rates in cases with available follow-up data[a]

		Cancer-specifc survival (*n*=102)			Disease-free interval (*n*=95)		
		n	Median survival (days)	Average 1-year survival rate *n* (%)	*n*	Median survival (days)	Average 1-year survival rate *n* (%)
Age	≤10 years	78	1.700	70 (68.63%)	78	1.674	66
	>10 years	24	694	14 (13.73%)	24	668	(69.47%)
							13 (13.68%)
	P		0.0018			0.0046	
Weight	≤10 Kg	60	1.498	51 (50%)	60	902	47
	>10 Kg	42	1.473	28 (27.45%)	42	1.432	(49.47%)
							33 (34.74%)
	P		0.1949			0.1333	
Pseudocyesis	Absent	86	1.555	71 (69.61%)	86	1.528	66 (69.47%)
	Present	16	1.612	9 (8.82%)	16	1.612	9 (9.47%)
	P		0.3573			0.3113	
Hormone administration	No	80	1.451	67 (65.69%)	80	1.378	63 (66.32%)
	Yes	22	1.469	18 (17.65%)	22	1.462	17 (17.89%)
	P		0.4608			0.5470	
Clinical Stage (TNM)	I	57	1.632	51 (50%)	57	1.282	49
	II	17	1.268	13 (12.75%)	17	1.217	(51.58%)
	III	20	1.294	17 (16.67%)	20	1.234	12
	IV	8	413	2 (1.96%)	9	402	(12.63%)
	P		0.0007			0.0016	15 (15.79%)
							2 (2.11%)
Surgical technique	Lumpectomy	5	1.484	3 (2.94%)	5	1.192	3 (3.16%)
	Regional mastectomy	34	1.464	26 (25.49%)	34	1.154	28 (29.47%)
	Radical mastectomy	51	1.471	40 (39.22%)	51	1.448	38 (40%)
	Combination of techniques	12	1.034	11 (10.78%)	12	1.034	11 (11.58%)
	P		0.6021			0.4172	
Tumor size	T1 (<2cm)	61	1.566	53 (51.96%)	61	1.234	51 (53.68%)
	T2 (2 – 3cm)	17	1.268	13 (12.75%)	17	1.217	12 (12.63%)
	T3 (>3cm)	24	1.248	19 (18.63%)	24	1.199	17 (17.89%)
	P		0.0064			0.0133	
Histological type	Mixed tumor carcinoma	61	1.528	56 (54.9%)	61	1.460	52 (54.74%)
	Solid carcinoma	10	1.019	5 (4.9%)	10	1.019	9 (9.47%)
	Tubular carcinoma	11	1.614	5 (4.9%)	11	1.238	5 (5.26%)
	In situ carcinoma	1	365	0	1	365	0
	Pleomorphic lobular	2	410	0	2	365	0
	carcinoma	4	180	0	4	180	0
	Micropapillary carcinoma	1	---	0	1	---	0
	Mucinous carcinoma	12	331	9 (8.82%)	12	110	9 (9.47%)
	Papillar carcinoma						
	P		0.0027			0.0055	
Histological grade	Grade I	42	1.470	36 (35.29%)	42	1.454	35
	Grade II	42	1.600	39 (38.24%)	42	1.254	(36.84%)
	Grade III	18	1.023	8 (7.84%)	18	986	36
							(37.89%)
							9 (9.47%)
	P		0.0004			0.0010	
Number of tumors	Single	30	1.308	23 (22.55%)	30	1.023	21 (22.11%)
	Multiple	72	1.663	62 (60.78%)	72	1.639	59 (62.11%)
	P		0.1749			0.1697	

*Represents the mean of variables which maedian has not yet been determined.

[a]Survival values are percentages for cumulative survival determined by Log-Rank test. Significance level adopted was <0.05.

The time-dependent proportional hazards model, based on the Cox proportional hazards model, was significant for CSS and DFI in the analysis between the OVH vs Non-OVH groups. Animals in the OVH group had higher means of CSS and DFI (1923 and 1919 days) and those in the Non-OVH group had lower averages of CSS and DFI (1238 and 1147 days, respectively) (P=0.0014; P=0.0011; Figures 3A and B).

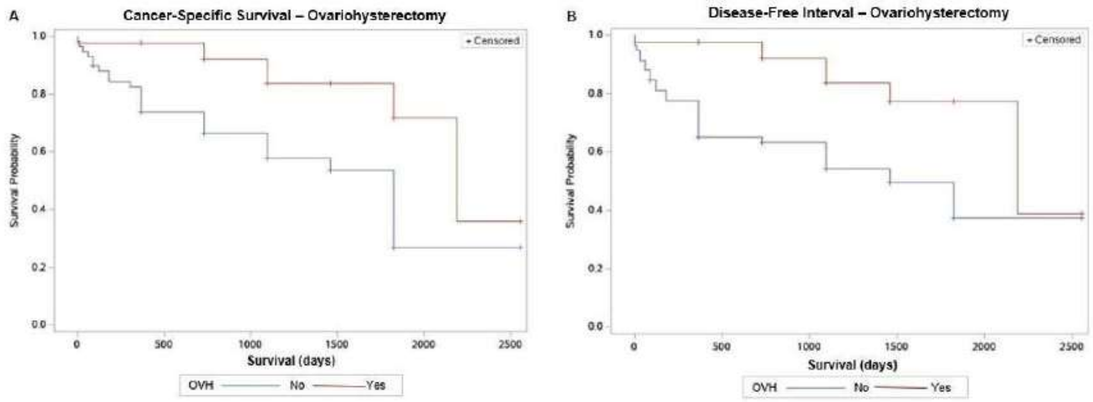
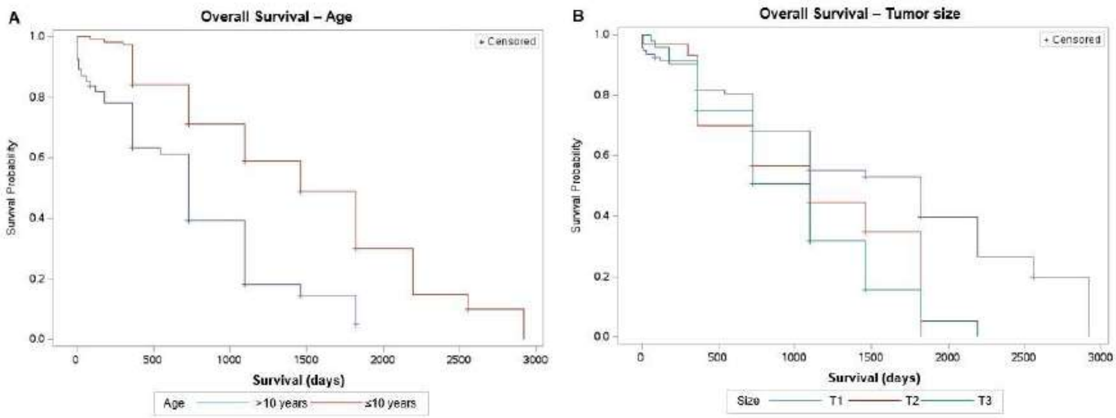


Figure 3. Kaplan–Meier cancer-specific survival and disease-free interval curve of female dogs with mammary carcinomas investigating proportional time dependent risk between the OVH and Non-OVH groups for CSS (Figure 3A) and DFI (Figure 3B).

5.3.2.2. Overall survival (OS)

A significant association was observed between age, tumor size, clinical stage, histological classification of tumors and histological grade for OS (P=<.0001, Figure 4A; P=0.0030, Figure 4B; P=0.0003, Figure 4C; P=0.0421, Figure 4D; P=0.0059, Figure 4E, respectively).



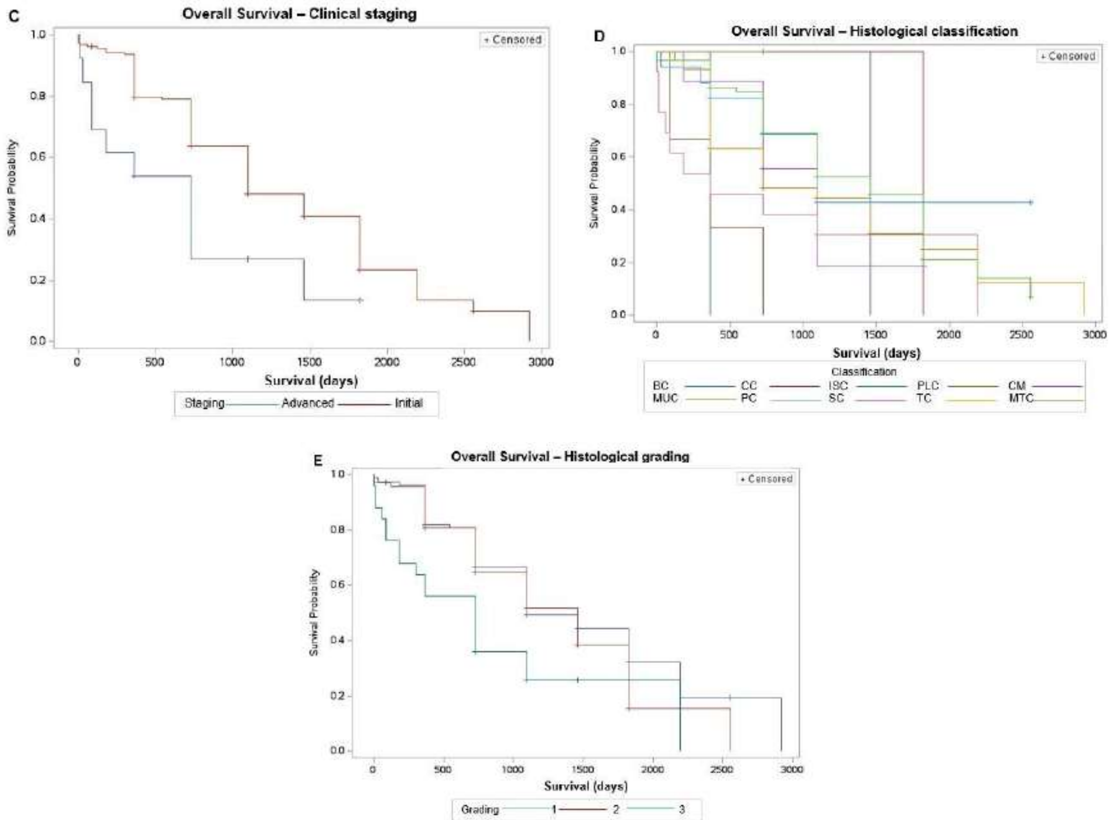


Figure 4. Kaplan–Meier overall survival curve of female dogs with mammary carcinomas investigating the prognostic effect of ovariohysterectomy (OVH) performed at the time of tumor removal in OS. MAmmary carcinomas classified according to: A – age: ≤10 years (mean 1,521 days) and >10 years (mean 788 days). B – Tumor size: T1 (mean 1588 days), T2 (mean 1102 days) and T3 (mean 997 days). C – Clinical stage: I (mean 1657 days), II (mean 1141 days), III (mean 1011 days), and IV (648 days). D – Histological classification: ISC - *in situ* carcinoma (mean 365 days), PLC - pleomorphic lobular carcinoma (mean 395 days), MC - micropapillary carcinoma (mean 871 days), MUC - mucinous carcinoma (mean 0 days), PC - papillar carcinoma (mean 892 days), SC - solid carcinoma (mean 869 days), TC - tubular carcinoma (mean 1215 days), MTC - mixed tumor carcinoma (mean 1404 days), BC - basaloid carcinoma (mean 1460 days) and CC - cribriform carcinoma (mean 1825 days). E – Histological grade: I (mean 1488 days), II (1329 days) and III (887 days), respectively.

OS was not influenced by weight, hormone administration, pseudocyesis, number of tumors and the type of surgical procedure performed. OS information for mammary carcinomas in investigating the effect of ovariohysterectomy (OVH group) or to remain intact (Non-OVH group) during tumor removal is detailed in Table 4.

Table 4. One-year corrected survival rates in cases with available follow-up data[a]

		Overall survival (*n*=171)		
		n	Median survival (days)	Average 1-year survival rate *n* (%)
Age	≤10 years	116	1.521	30 (17.54%)
	>10 years	55	788	20 (11.69%)
	P		<.0001	
Weight	≤10 Kg	102	1.364	29 (16.95%)
	>10 Kg	69	1.226	26 (15.2%)
	P		0.7866	
Pseudocyesis	Absent	147	1.249	44 (25.73%)
	Present	24	1.624	4 (2.33%)
	P		0.1600	
Hormone administration	No	139	1.271	46 (26.90%)
	Yes	32	1.404	9 (5.26%)
	P		0.6139	
Clinical Stage (TNM)	I	86	1.657	11 (6.43%)
	II	28	1.141	11 (6.43%)
	III	44	1.011	9 (5.26%)
	IV	13	648	3 (1.75%)
	P		0.0003	
Surgical technique	Lumpectomy	10	1.048	4 (2.33%)
	Regional mastectomy	61	1.393	14 (8.18%)
	Radical mastectomy	82	1.237	17 (9.94%)
	Combination of techniques	18	1.556	4 (2.33%)
	P		0.5648	
Tumor size	T1 (<2cm)	93	1.588	24 (14.03%)
	T2 (2 – 3cm)	30	1.102	11 (6.43%)
	T3 (>3cm)	48	997	10 (5.84%)
	P		0.0030	
Histological type	Mixed tumor carcinoma	97	1.404	20 (11.69%)
	Solid carcinoma	13	869	5 (2.92%)
	Tubular carcinoma	30	1.215	11 (6.43%)
	In situ carcinoma	1	365	0
	Pleomorphic lobular	3	395	1 (0.58%)
	carcinoma	9	871	5 (2.92%)
	Micropapillary carcinoma	1	---	0
	Mucinous carcinoma	17	892	5 (2.92%)
	Papillar carcinoma	1	1.460	0
	Basaloid carcinoma	1	1.825	0
	Cribriform carcinoma			
	P		0.0421	
Histological grade	Grade I	68	1.488	17 (9.94%)
	Grade II	78	1.329	15 (8.77%)
	Grade III	25	887	9 (5.26%)
	P		0.0059	
Number of tumors	Single	41	1.144	13 (7.6%)
	Multiple	130	1.345	42 (24.56%)
	P		0.7671	

[a]Survival values are percentages for cumulative survival determined by Log-Rank test. Significance level adopted was <0.05.

5.3.3. Multivariate survival analysis

5.3.3.1. Cancer-specifc survival (CSS) and Disease-free interval (DFI)

The multivariate Cox regression model was used to predict the probability of CSS and DFI for at least one year after surgery and included the explanatory variables: breed (mixed or purebred), OVH (yes or no), pseudocyesis (present or absent), TNM-based clinical stage (initial or advanced), tumor size (score 1-3) ,surgical technique (lumpectomy, regional mastectomy, radical mastectomy or combination of techniques), histological grade (score 1-3), hormone administration (yes or no), age (years) and weight (kg). The model was able to significantly explain the variability observed in the population (P<0.05). For CSS only the variables histological grade, age and weight remain as prognostic factors in the final model (Table 4) and for DFI only the variables histological grade, age, weight and Non-OVH remain as prognostic factors in the final model (Table 5).

Table 5. Analysis of maximum likelihood estimates of multivariate logistic regression to predict the probability of CSS and DFI for at least 1 year after surgery in 102 dogs affected by mammary tumor.

Cancer-specifc survival (CSS)				**Disease-free interval (DFI)**			
Variables	**Estimate**	**Standard Error**	***P***	**Variables**	**Estimate**	**Standard Error**	***P***
Intercept	10.9479	2.9795	0.0002	Intercept	10.8295	2.8495	0.0001
Age	-0.6551	0.2016	0.0012	Idade	-0.6412	0.1923	0.0009
Weight	-0.1324	0.0581	0.0226	Peso	-0.1258	0.0575	0.0286
Grade (1)	-0.0789	0.8601	0.9269	Grade (1)	-0.4763	0.6729	0.4791
Grade (2)	2.2551	1.0994	0.0403	Grade (2)	1.9905	0.8408	0.0179
–	–	–	–	Não-OVH	-1.6837	0.7054	0.0170
c (Area under ROC curve) = 0.956.				c (Area under ROC curve) = 0.942.			

The multivariate Cox regression model was also used to investigate the effect of ovariohysterectomy at the time of mastectomy for CSS and DFI and included the explanatory variables: age (≤10 years or >10 years), weight (≤10 Kg or >10 Kg), pseudocyesis (present or absent), number of tumors (single or multiple), OVH (yes or no), TNM-based clinical stage (initial or advanced), tumor size (score 1-3), surgical technique (lumpectomy, regional mastectomy, radical mastectomy or combination of techniques), histological grade (score 1-3), hormone administration (yes or no). The model was able to significantly explain the variability observed in the population (P<0.05). For both CSS and DFI the Age variable (>10 years), Histological grade and Non-OVH remain as prognostic factors in the final model (Table 6).

Table 6. Multivariable Cox regression results analyzing factors that influence CSS and DFI in 102 canine females with mammary carcinomas in the investigation of the prognostic effect of ovariohysterectomy at the time of mastectomy.

Cancer-specifc survival (CSS)						
Variables	**Estimate**	**Standard Error**	***P***	**Hazard Ratio**	**95% Confidence interval**	
Age (>10 years)	1.05273	0.39577	0.0078	2.865	1.319	6.224
Grade (2)	-0.49380	0.44389	0.2659	0.610	0.256	1.457
Grade (3)	1.10637	0.43314	0.0106	3.023	1.294	7.066
Non-OVH	1.34811	0.43872	0.0021	3.850	1.629	9.097
Disease-free interval (DFI)						
Variables	**Estimate**	**Standard Error**	***P***	**Hazard Ratio**	**95% Confidence interval**	
Age (>10 years)	0.87571	0.39438	0.0264	2.401	1.108	5.200
Grade (2)	-0.41164	0.44782	0.3580	0.663	0.275	1.594
Grade (3)	0.99897	0.43509	0.0217	2.715	1.157	6.371
Non-OVH	1.31873	0.43556	0.0025	3.739	1.592	8.779

5.3.3.2. Overall survival (OS)

The multivariate Cox regression model was used to predict the likelihood of OS for at least one year after surgery and included the explanatory variables: OVH (yes or no), pseudocyesis (present or absent), clinical stage based on TNM (initial or advanced), tumor size (score 1-3), surgical technique (lumpectomy, regional mastectomy, radical mastectomy or combination of techniques), histological grade (score 1-3), hormone administration (yes or no), age (≤10 years and >10 years), weight (kg) and number of mammary lesions (single or multiple). The model was able to significantly explain the variability observed in the population ($P<0.05$). For OS only the Age variable (>10 years), Histological grade and Non-OVH remain as prognostic factors in the final model (Table 7).

Table 7. Multivariate Cox regression results analyzing factors that influence OS in 171 female dogs with mammary carcinomas in the investigation of the prognostic effect of ovariohysterectomy at the time of mastectomy.

Overall survival (OS)						
Variables	**Estimate**	**Standard Error**	***P***	**Hazard Ratio**	**95% Confidence interval**	
Age (>10 years)	0.90055	0.20962	<.0001	2.461	1.632	3.711
Grade (2)	0.12887	0.22554	0.5677	1.138	0.731	1.770
Grade (3)	0.87938	0.28979	0.0024	2.409	1.365	4.252
Non-OVH	0.89751	0.23464	0.0001	2.453	1.549	3.886

5.4. Discussion

Estrogen has been recognizedas a major factor in mammary carcinogenesis in women and dogs. In both species, the risk of breast cancer is directly correlated with the duration of exposure of breast tissue to bioavailable estrogens (28, 29). In this study, the effect of OVH at the same time as the mastectomy was evaluated in a mixed population of female dogs affected by spontaneous mammary carcinomas at different clinical stages and it was possible to observe that the variables age (>10 years), histological grade (3) and Non-OVH are independent prognostic factors and significantly interfere with the cancer-specific survival (CSS), overall survival (OS) time, and disease-free interval (DFI) of these patients.

Most previous studies focused on CMT patients disease-free survival and overall survival only (13, 18, 19, 20, 30). Thus, the present study is of particular interest due to the cutoff of CMTs described to date, making it a rare report on disease-free interval, cancer-specific survival, and overall CMT survival.

The effect of spaying status on CMTs has been widely investigated, with contradictory results (31). The positive effect of gonadectomy at the time of mastectomy was found in non-neoplastic lesions (hyperplastic/dysplastic), benign mammary neoplasms (30) and in female dogs with grade II carcinomas presenting estrogen receptors or with increased serum concentrations of 17β-estradiol (18). Thus, the histological classification and grading of tumors is directly related to the hormonal influence on the lesions, directly affecting the patient's survival time, as shown also in our study.

Kristiansen et al. (18) did not find significant benefits of hormonal ovarian ablation in dogs with mammary carcinomas in DFI or OS. As well as, Banchi et al. (20) and Burrai et al. (19), showing that castration status had no influence on benign or malignant tumors. These results disagree with our findings, where CSS, OS and DFI were significantly influenced by castration status (non-OVH group) at the time of mammary carcinoma removal and we also observed that not all dogs with CMTs benefit from OVH at the time tumor removal, corroborating Kristiansen et al. (18) and Bohrer, Löhr and Kutzler (32).

In the present study intact dogs developed greater recurrence when compared to animals submitted to adjuvant therapy in the OVH group (36.21% versus 27.27%), these results are consistent with those reported by Sorenmo et al. (33). Continued exposure to estrogen after tumor removal contributes to increased risk of new breast tumors and supports a procarcinogenic action of the estrogen effect (30). This data also supports the recommendation of OVH together with tumor removal for its benefit on decreasing the chance of tumor recurreance together with CSS, DFI and OS.

The incidence of CMTs in purebred animals were higher than in mixed breed female dogs and the frequencies are consistent with previously reported information (3, 20, 21, 33, 34).

Furthermore, it was observed that the use of hormones did not interfere with CSS, OS and DFI, which differs from what was expected, since the prolonged use of synthetic progestins can increase the incidence of CMTs (35, 36). Pseudocyesis is implicated in the development of CMTs (37 38, 39), although in this study it did not demonstrate any significant effect.

According to other studies (19, 26, 40, 41), tumor size is another important clinical parameter with prognostic value, in line with our findings, where tumor size was shown to be an independent prognostic factor for CSS, OS and DFI of CMTs.

Mammary cancer risk prediction models can help female dogs and their health care providers in making decisions about screening and surgical treatment associated with OVH as adjunctive therapy. However, an accurate estimate of the risk of developing mammary tumors is critical for calculating the risk-benefit ratio for these patients. Thus, more clinical studies should be carried out to adapt these concepts to veterinary oncology practice Because of this, we emphasize that spaying at the time of mastectomy should be considered in intact female dogs, but it is should be analysed for each species as it can be species-especific and other significant variables should be considered, such as age and histological grade of the tumor, which also reduced CSS, OS and DFI of the animals in this study.

5.5. Conclusion

This study reveals that, for the treatment of mammary gland tumors, ovariohysterectomy together with surgical excision of the nodules is an effective adjuvant therapy prolonging disease-free is an effective adjuvant therapy in the surgical treatment of the tumor and that its performance is an important factor influencing the survival time of female dogs diagnosed with mammary gland carcinoma. We emphasize that not all animals will benefit from OHV and that its performance should be recommended mainly for young to middle-aged animals with grade I and II mammary carcinomas confirmed by histopathological examination, these factors must be taken into account for the eligibility of the procedure by the surgeon.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Conflict of interest statement

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

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Author Contribution Statement

ESS, CCR and VRO conducted laboratory analyses and data interpretation; ESS wrote the article; ESS, CCR and TBS drafted and revised the article draft; JVFM and FLDO translated the paper; PTR, FAV and ECCR revised the manuscript draft and supervised the laboratory work. ESS, CCR and APBB planned and designed the study. All authors read and approved the final version of the article.

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