

UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
Programa de Pós-graduação em Patologia

MAYRA CUNHA FLECHER

AVALIAÇÃO DO PAPEL PROGNÓSTICO DE NEUTRÓFILOS E LINFÓCITOS EM
NEOPLASIAS DE ARRANJO SÓLIDO DA GLÂNDULA MAMÁRIA CANINA

Belo Horizonte

2024

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Tese apresentada ao Programa de Pós-graduação em
Patologia da Universidade Federal de Minas Gerais como
requisito para obtenção do título de Doutor em Patologia

Orientador: Dr. Geovanni Dantas Cassali

Co-orientadora: Dra. Karen Yumi Ribeiro Nakagaki

Belo Horizonte

2024

043

Flecher, Mayra Cunha.

Avaliação do papel prognóstico de neutrófilos e linfócitos em neoplasias de arranjo sólido da glândula mamária canina [manuscrito] / Mayra Cunha Flecher. – 2024.

118 f. : il. ; 29,5 cm.

Orientador: Dr. Geovanni Dantas Cassali. Co-orientadora: Dra. Karen Yumi Ribeiro Nakagaki.

Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas. Programa de Pós-Graduação em Patologia.

1. Patologia. 2. Neoplasias Mamárias Animais. 3. Estrogênios. 4. Inflamação. 5. Imuno-Histoquímica. 6. Metástase Neoplásica. I. Cassali, Geovanni Dantas. II. Nakagaki, Karen Yumi Ribeiro. III. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. IV. Título.

CDU: 616



UNIVERSIDADE FEDERAL DE MINAS GERAIS

INSTITUTO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA DA UFMG

FOLHA DE APROVAÇÃO

***“Avaliação do Papel Prognóstico de Neutrófilos e
Linfócitos em Neoplasias de Arranjo Sólido da Glândula
Mamária Canina”***

MAYRA CUNHA FLECHER

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de **Pós-Graduação em Patologia**, como requisito para obtenção do grau de **Doutor em PATOLOGIA**, área de concentração **PATOLOGIA INVESTIGATIVA**.

Aprovada em 09 de julho de 2024, pela banca constituída pelos membros:

Profa. Dra. Renee Laufer Amorim, UNESP - Botucatu

Profa. Dra. Lidianne Narducci Monteiro, UVV

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Belo Horizonte, 09 de julho de 2024.



Documento assinado eletronicamente por **Geovanni Dantas Cassali, Professor do Magistério Superior**, em 19/07/2024, às 14:11, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



Documento assinado eletronicamente por **Keren Yumi Ribeiro Nakagaki, Usuária Externa**, em 23/07/2024, às 07:46, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



Documento assinado eletronicamente por **Igor Luiz Salardani Senhorello, Usuário Externo**, em 09/08/2024, às 16:24, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



Documento assinado eletronicamente por **Lidianne Narducci Monteiro, Usuária Externa**, em 21/08/2024, às 11:14, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



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Dedico esse trabalho à minha família, meu filho Breno, meu esposo e aos meus pais, que me deram todo apoio na busca de um objetivo profissional e satisfação pessoal.

AGRADECIMENTOS

Durante esses quatro anos foram muitas idas e vindas, muito voos, muitas estradas, noites mal dormidas, dias madrugando, correria para dar conta de tarefas do doutorado que se misturavam com afazeres do trabalho, provas, aulas, e a família no meio de tudo isso. Portanto, em todo esse período são muitas pessoas para agradecer.

Agradeço ao meu filho, que se manteve bem durante a minha ausência, mesmo sem entender muito o que todas essas idas e vindas significavam, sabia que eu estava estudando e que isso era importante para mim. Agradeço de forma especial ao meu esposo, Cristian, que cuidou da nossa família quando eu estava ausente, me deu força e apoio para não desistir.

Aos meus pais e a minha irmã que me apoiaram durante toda a minha vida acadêmica, e me inspiraram no caminho do bem e da ética.

Ao professor Geovanni, que aceitou me orientar, mesmo sabendo que eu estaria a alguns quilômetros de distância. Agradeço imensamente a oportunidade e a confiança. Além de professor, foi um amigo durante todos esses anos. Desejo manter a amizade, e a cooperação profissional.

Gratidão aos que me incentivaram a iniciar o doutorado, quando eu achava que não seria possível, Tayse Domingues, Breno Salgado e Rodrigo Horta.

Obrigada aos amigos e familiares por cada palavra de incentivo quando eu desanimava, ou quando achava que não ia dar conta, pois as cobranças do trabalho se misturavam com as do doutorado e as pessoais. São muitos os amigos.... Maylla, Halana, Emy, Lidianne, Caroline Saraiva, Roberta Flecher, Fernanda, Igor, Fernando, Vanderleia.

Agradeço aos meus alunos, principalmente os de residência e de iniciação científica que me auxiliaram nas coletas de materiais e de dados.

E não posso esquecer dos novos amigos que fiz em Belo Horizonte, as meninas do LPC e do Celulavet. Sou grata por cada risada (de alegria e de desespero), pelas conversas, pelos cafés e pão de queijo, e o auxílio durante a execução da pesquisa. Quero agradecer em especial a Fernanda Rezende que me hospedou muitas vezes, e essa amizade ficará para sempre.

Este trabalho foi realizado no Laboratório de Patologia Comparada (LPC) do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Minas Gerais.

Apoio financeiro: CNPq.



RESUMO

O estudo morfológico e imuno-histoquímico das neoplasias mamárias caninas denominadas carcinomas sólidos mostrou que elas podem ser compostas por células epiteliais e/ou mioepiteliais, caracterizando diferentes tipos histológicos. Nas cadelas, o aumento da inflamação é associado a maior graduação histológica, metástases e invasão linfática. Porém, ainda não se conhece as características imunofenóticas e a importância da inflamação na progressão desses tipos histológicos. O objetivo deste estudo é caracterizar a intensidade do infiltrado inflamatório, principalmente linfócitos e neutrófilos, e os imunofenótipos nas neoplasias com arranjo sólido da glândula mamária canina, e associar a fatores prognósticos (metástase para linfonodo, contagem mitótica e índice de Ki-67 e tamanho tumoral), expressão de ciclo-oxigenase (Cox-2), necrose tumoral e sobrevida global e específica das cadelas. De 120 casos de neoplasias mamárias diagnosticadas como carcinomas sólidos e basaloídes, foram selecionadas 65, reclassificados como carcinoma com padrão sólido, carcinoma basaloide (BC), carcinoma papilar sólido, adenomioepitelioma maligno (MAME) e mioepitelioma maligno (MME). A imuno-histoquímica foi realizada para determinar os imunofenótipos (luminal A, luminal B HER-2 negativo e HER-2 positivo, HER-2 superexpresso e triplo negativo), identificar a expressão da proteína Ki-67, de Cox-2 e marcação de linfócitos CD3⁺ e CD79⁺. A graduação do infiltrado inflamatório peritumoral e intratumoral foi realizada nas lâminas histológicas através de um escore (distribuição x intensidade da inflamação) que estabeleceu uma graduação de I a III. Posteriormente, fez-se a contagem de linfócitos e neutrófilos infiltrantes de tumor (TILs e TANs) em 8 campos *hot spots* (600x), e a de linfócitos CD3⁺ e CD79⁺ em 8 campos *hot spots* (400x). A partir daí a mediana para cada tipo celular, na histologia e na imuno-histoquímica, foi estabelecida como ponto de corte para dicotomizar as neoplasias em alta e baixa intensidade de infiltrado inflamatório. A maioria das neoplasias de arranjo sólido apresentou imunofenótipo Luminal B HER-2 negativo, mas o MME e o MAME tiveram significativamente maior ocorrência de Luminal A quando comparado aos outros tipos histológicos. A graduação da inflamação peri e intratumoral não teve correlação estatística com os tipos histológicos, imunofenótipos, índice de Ki-67 e tamanho tumoral. BCs tiveram significativamente baixa intensidade de TILs intratumoral, comparado aos outros tipos histológicos, e neoplasias luminais B HER-2 positivo e negativo apresentaram alta intensidade de linfócitos T CD3⁺ intratumoral. O aumento do grau de inflamação peri e intratumoral, e alta intensidade de linfócito B CD79⁺ intratumoral foram estatisticamente associados à metástase para linfonodo. A contagem mitótica foi proporcional a intensidade de TILs e linfócitos BCD79⁺ intratumorais. Já a porcentagem de Ki-67 foi proporcional a intensidade de TILs, linfócitos T CD3⁺ e B CD79⁺ peritumorais. A expressão Cox-2 não foi associada ao grau de inflamação, a intensidade de TILs, e seus subtipos, e de TANs. Apesar do grau de inflamação não ter sido associado à sobrevida das cadelas, a alta intensidade de TANs peritumoral foi associada a menor sobrevida global, e a alta intensidade de TILs e linfócitos T CD3⁺ intratumorais foram associadas a menor sobrevida específica. A metástase para linfonodo aumenta o risco de morte das cadelas pela neoplasia, sendo essa a única variável que se manteve como fator independente para o risco de óbito das cadelas com neoplasias de arranjo sólido. Conclui-se que existe uma relação dos tipos histológicos com os imunofenótipos, e imunofenótipos luminais B apresentam maior intensidade de linfócitos T CD3⁺ intratumoral. A infiltração intratumoral apesar de estar associada a alguns fatores prognósticos e a redução do tempo de vida, não é um fator independente. Um dos principais fatores influenciados pela inflamação é a presença de metástase para linfonodo, que é considerado um fator prognóstico independente neste estudo.

Palavras-chave: TILs, estrógeno, inflamação, imuno-histoquímica, metástase

ABSTRACT

The morphological and immunohistochemical study of canine mammary neoplasms referred to as solid carcinomas showed that they can be composed of epithelial and/or myoepithelial cells, characterizing different histological types. In female dogs, greater inflammation is associated with higher histological grade, metastasis, and lymphatic invasion. However, the immunophenotypic characteristics and the importance of inflammation in the progression of these histological types are still unknown. The aim of this study is to characterize the intensity of inflammation, mainly lymphocytes and neutrophils, and the immunophenotypes in neoplasms with a solid arrangement of the canine mammary gland, and to associate them with prognostic factors (lymph node metastasis, mitotic count, Ki-67 index, and tumor size), cyclooxygenase (Cox-2) expression, tumor necrosis, and overall and specific survival of the female dogs. Out of 120 cases of mammary neoplasms diagnosed as solid and basaloid carcinomas, 65 were selected and reclassified as carcinoma with a solid pattern, basaloid carcinoma (BC), solid papillary carcinoma, malignant adenomyoepithelioma (MAME), and malignant myoepithelioma (MME). Immunohistochemistry was performed to determine the immunophenotypes (luminal A, luminal B HER-2 negative and HER-2 positive, HER-2 overexpressed, and triple negative), identify the expression of the Ki-67 protein, Cox-2, and labeling of CD3⁺ and CD79⁺ lymphocytes. The grading of the peritumoral and intratumoral inflammatory infiltrate was performed on histological slides using a score (distribution x intensity) that established a grade from I to III. Subsequently, the count of tumor-infiltrating lymphocytes and neutrophils (TILs and TANs) was performed in 8 hotspot fields (600x), and the count of CD3⁺ and CD79⁺ lymphocytes in 8 hotspot fields (400x). From this, the median for each cell type, in histology and immunohistochemistry, was established as a cut-off point to dichotomize the neoplasms into high and low intensity inflammatory infiltrate. Most of the solid arrangement neoplasms presented luminal B HER-2 negative immunophenotype, but MME and MAME had a significantly higher occurrence of luminal A compared to the other histological types. The grading of peri and intratumoral inflammation had no statistical correlation with histological types, immunophenotypes, Ki-67 index, and tumor size. BCs had significantly low intensity of intratumoral TILs compared to other histological types, and luminal B HER-2 positive and negative neoplasms showed high intensity of intratumoral TCD3⁺ lymphocytes. The increase in peri and intratumoral inflammation grade, and high intensity of intratumoral BCD79⁺ lymphocytes were statistically associated with lymph node metastasis. Mitotic count was proportional to the intensity of intratumoral TILs and BCD79⁺ lymphocytes. The Ki-67 percentage was proportional to the intensity of peritumoral TILs, TCD3⁺ lymphocytes, and BCD79⁺ lymphocytes. Cox-2 expression was not associated with the grade of inflammation, TILs intensity, and their subtypes, and TANs. Although the grade of inflammation was not associated with the survival of the female dogs, high intensity of peritumoral TANs was associated with lower overall survival, and high intensity of intratumoral TILs and T CD3⁺ lymphocytes were associated with lower specific survival. Lymph node metastasis increases the risk of death from neoplasia in female dogs, being the only variable that remained as an independent factor for the risk of death in female dogs with solid arrangement neoplasms. It is concluded that there is a relationship between histological types and immunophenotypes, and luminal B immunophenotypes present higher intensity of intratumoral TCD3⁺ lymphocytes. Intratumoral infiltration, although associated with some prognostic factors and reduced survival time, is not an independent factor. One of the main factors influenced by inflammation is the presence of lymph node metastasis, which is considered an independent prognostic factor in this study.

Keywords: lymphocytes, estrogen, inflammation, immunohistochemistry, metastasis

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LISTA DE ABREVIATURAS E SIGLAS

TIL – Linfócitos infiltrantes tumoral

TAN – Neutrófilos infiltrantes tumoral

SRD – Sem Raça Definida

RE – Receptor de Estrógeno

RP – Receptor de Progesterona

HER – Receptor do Fator de crescimento Epidermal Humano

TN – Triplo negativo

SMA – Actina de Músculo Liso

CK - Citoqueratina

Cox– Ciclo-oxigenase

AFIP - *Armed Forces Institute of Pathology*

LT – Linfócitos T

NK – *Natural Killer*

CSP – Carcinoma com padrão sólido

CB – Carcinoma basaloide

CPS – Carcinoma papilar sólido

ADM – Adenomioepitelioma maligno

MM – Mioepitelioma maligno

MHC – Complexo de histocompatibilidade

IL- Interleucina

VEGF – Fator de crescimento de células endoteliais

CSF – Fator estimulador de colônias

MMP – metaloproteases

TNF – Fator de necrose tumoral

IFN - Interferon

CAA – Célula apresentadora de antígeno

DAB - Diaminobenzidina

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1. INTRODUÇÃO

As neoplasias mamárias são os tumores mais frequentes nas cadelas (Cassali et al., 2014; Pastor et al., 2018; Rodrigues et al., 2023), com predomínio das neoplasias malignas na maior parte dos estudos (Toribio et al., 2012; Rasotto et al., 2017; Silva et al., 2019). Cadelas idosas e não castradas apresentam maior ocorrência dessa doença (Gedon et al., 2021; Nunes et al., 2018; Rodriguez et al., 2022).

Fatores prognósticos importantes avaliados nas cadelas com neoplasias mamárias são o tamanho tumoral, metástase para linfonodos e à distância, tipo e graduação histológica, e contagem mitótica (Rasotto et al., 2017, Pastor et al., 2018; Nunes et al., 2018). Além desses, as identificações de outros fatores estabelecidos através da marcação imuno-histoquímica também são importantes como índice de proliferação celular (Ki-67) e receptores hormonais (Pastor et al., 2018; Cassali et al., 2020, Nunes et al., 2022). A classificação histológica das neoplasias mamárias nas cadelas é ainda uma das formas mais importantes de estabelecimento do prognóstico, sendo o carcinossarcoma, carcinoma micropapilar e carcinomas sólidos os de pior prognóstico (Nunes et al., 2018; Seung et al., 2021). O carcinoma sólido, previamente caracterizado por Misdorp (1999), foi reclassificado e subdividido por Goldschmidt et al. (2011), e após estudos morfológico e imuno-histoquímicos novas propostas de classificação e subdivisão foram feitas, separando os tipos epiteliais dos que apresentam proliferação mioepitelial associada ou como população única (Yoshimura et al., 2014; Nakagaki et al., 2022). Baseado nisso, recentemente foi proposto a divisão dos carcinomas sólidos em carcinoma basaloide, carcinoma papilar sólido, carcinoma com padrão sólido, adenomioepitelioma maligno, mioepitelioma maligno e carcinoma neuroendócrino (Nakagaki et al., 2022).

Os imunofenótipos por avaliação imuno-histoquímica ou molecular, apesar de serem incluídos nos fatores prognósticos para as mulheres, ainda estão sendo investigados na veterinária (Abadie et al., 2018; Nunes et al., 2022). Na medicina veterinária, tem sido proposto a definição de imunofenótipos das neoplasias mamárias para trazer informações adicionais ao tipo histológico e melhorar o entendimento do comportamento biológico dessas neoplasias. Entretanto, não existe um consenso que defina o protocolo para fazer essas avaliações, e portanto, os trabalhos são realizados com metodologias distintas (Sassi et al., 2010; Im et al., 2014; Nunes et al., 2022). Mesmo com essas diferenças nos protocolos, através da análise imuno-histoquímica, neoplasias com imunofenótipos luminais são os mais comuns em cadelas (Gama et al., 2008; Varallo et al., 2019; Nunes et al., 2022).

A inflamação associada ao câncer pode ter um papel pró ou antitumoral dependendo da composição do microambiente (células imunes, secreção de citocinas e quimiocinas, fatores de crescimento e componentes da matriz extracelular), que é diretamente influenciada pelo estágio de crescimento da neoplasia (Jakubowska et al., 2017; Shrihari, 2017; Nguyen et al., 2018). À medida que a neoplasia progride, há maior quantidade de células como linfócitos TCD4⁺ helper 2 (h2), diferenciação de linfócitos reguladores, células supressoras mielóides, macrófagos e neutrófilos (M2 e N2). Essas células secretam citocinas e vários tipos de fatores que induzem angiogênese, aumentam a expressão de genes associado à transição epitélio-mesênquima, além de reduzir a resposta anti-tumoral (Zhu et al, 2015; Yu et al., 2016; Amens et al, 2021; Kwantwia et al., 2021).

Em mulheres sabe-se que a intensidade e a distribuição do infiltrado inflamatório, principalmente os linfócitos infiltrantes do tumor (TILs), tem associação com características morfológicas tumorais e sobrevida das pacientes. Além disso, também é conhecido que existe uma relação do imunofenótipo da neoplasia com a intensidade do TIL estromal e seus subtipos, pois neoplasias com receptores hormonais negativos e que expressam HER-2 ou são TN, apresentam maior porcentagem de TILs (Lee et al, 2015; Koletsa et al, 2020; Gao et al, 2020).

Nas cadelas, os linfócitos são as células que predominam nas neoplasias mamárias, e estão em maior quantidade nos tumores malignos, com maior grau histológico, com invasão linfática (Kim et al., 2013) e metástase para linfonodo (Muscatello et al., 2023). Uma maior intensidade de linfócitos também foi associada à menor tempo de sobrevida (Estrela-lima et al., 2010). Porém, existem poucos estudos que correlacionam a inflamação entre os tipos histológicos (Estrela-lima et al., 2010; Souza et al., 2018), com os imunofenótipos (Kim et al., 2013; Abadie et al., 2018) e com outras características morfológicas como tamanho, metástase e índice proliferativo.

2. REVISÃO DE LITERATURA

2.1. Aspectos gerais das neoplasias da glândula mamária em cadelas

As neoplasias mamárias são muito comuns em cadelas e gatas, e no Brasil é o tipo neoplásico mais frequente nas cadelas (Rodrigues et al., 2023), podendo representar, nessa espécie, cerca de 50% a 70% de todos os cânceres (Horta et al., 2014; Pastor et al., 2018). As neoplasias malignas são predominantes em muitas casuísticas (Toribio et al., 2012; Rasotto et al., 2017; Silva et al., 2019), e eventualmente representam mais de 80% das neoplasias (Pastor et al., 2018; Nunes et al., 2018; Silva et al, 2023).

Nos estudos de casuísticas de neoplasias mamárias em cadelas há uma variação de frequência das raças dependendo da região ou país, mas estão entre as mais frequentes nos estudos nacionais o Poodle, Pinscher, Teckel (Silva et al., 2019; Rodrigues et al., 2023), em estudos internacionais são vistos Yorkshire, Teckel, Pastor Alemão (Kim et al., 2016; Litterine-Kaufman et al., 2019; Burrai et al., 2020). Além disso, os cães sem raça definida (SRD) também têm apresentado elevada casuística tanto no Brasil como em outros países (Silva et al., 2019; Rodrigues et al., 2023; Burrai et al., 2020; Gedon et al., 2021).

A idade, exposição prolongada a fatores hormonais e sobrepeso são fatores de risco associado ao desenvolvimento das neoplasias mamárias nas cadelas (Santos et al., 2020; Rodrigues et al., 2023; Silva et al., 2023). Neoplasias malignas são mais frequentes nas cadelas de meia idade a idosas com média 9 ou 10 anos de idade (Pastor et al., 2018; Gedon et al., 2021; Rodríguez et al., 2022). Burrai et al. (2020), observaram que a cada aumento de um ano da idade, há 12% mais chance da cadela desenvolver um tumor maligno. Também, na maioria dos estudos epidemiológicos sobre esse câncer nas cadelas, observa-se um predomínio de fêmeas não castradas (Nunes et al., 2018; Burrai et al., 2020, Seung et al., 2021), ocasionando uma prolongada exposição da glândula mamária aos hormônios sexuais (estrógeno e progesterona), o que favorece o desenvolvimento do câncer de mama. O primeiro estudo realizado por Schneider, em 1969, ainda é citado por muitas pesquisas, e afirma que a castração até os 6 meses reduz chance de desenvolvimento de câncer de mama para 0,5%, tendo esse benefício reduzido com o passar dos anos, e sendo quase nulo a partir de 4 anos de idade.

A avaliação macroscópica da neoplasia é um passo importante a ser realizado durante o exame clínico do paciente, pois algumas características podem sugerir o comportamento dessa neoplasia, como a localização, presença de nódulos únicos ou múltiplos, ulceração e tamanho (Sorenmo et al., 2020). A avaliação do tamanho tumoral, é uma das etapas do estadiamento do paciente e é considerado um fator prognóstico importante, pois sabe-se que tumores maiores comumente são malignos (Ferreira et al., 2009; Burrai et al., 2020; Gedon et al., 2021) e os paciente com tumores acima de 3cm apresentam menor tempo de sobrevida (Nunes et al., 2018). As neoplasias podem ser únicas ou múltiplas, e são mais comumente vistas nas mamas abdominais e inguinais (Nunes et al., 2018; Silva et al., 2019; Litterine-Kaufman et al., 2019). Além disso, cadelas com neoplasia múltiplas, também apresentam maior chance de pelo menos uma delas ser maligna (Litterine-Kaufman et al., 2019).

Microscopicamente, são muitos os tipos histológicos que podem ser diagnosticados na glândula mamária canina, pois as neoplasias podem ser de origem epitelial, mioepitelial e mesenquimal (Zapulli et al., 2021; Nakagaki et al., 2023). Além disso, diferentes tipos de

classificações histológicas das neoplasias mamárias caninas têm sido propostas. Uma das primeiras classificações foi a de Misdorp (1999) publicada pelo Instituto de Patologia das Forças armadas (AFIP), posteriormente vieram outras propostas como a de Goldschmidt et al (2011) e Cassali et al (2011, 2014). Dentre as neoplasias malignas, o carcinoma em tumor misto é um dos tipos histológicos mais diagnosticados, seguido do carcinoma papilar e o carcinoma tubular na classificação de Cassali et al. (2020) ou túbulo-papilar na classificação de Misdorp (1999). O carcinoma sólido também está entre os tipos mais frequentes de neoplasias mamárias nas cadelas (Nunes et al., 2018; Canadas et al., 2019; Silva et al., 2019; Rodrigues et al., 2023).

2.2 Neoplasias de arranjo sólido da glândula mamária canina

As neoplasias de arranjo sólido são as que apresentam um padrão de organização denso formando ninhos, cordões e trabéculas com fino estroma fibrovascular, e podem ser constituídas de células epiteliais e/ou mioepiteliais (Nakagaki et al., 2022; Nakagaki et al, 2023). Inicialmente essas neoplasias foram diagnosticadas como carcinoma sólido (Misdorp, 1999), e depois foram reclassificadas por Goldschmidt et al (2011) e subdividido em carcinoma sólido, carcinoma adenoescamoso e comedocarcinoma. Entretanto, algumas características histológicas e fenotípicas vistas nessas neoplasias, como a identificação de marcação nuclear da proteína p63 em neoplasias previamente classificadas como carcinoma sólido, geraram questionamentos quanto a utilização do termo “sólido” como um tipo histológico (Gama et al., 2003; Yoshimura et al., 2014). Assim foi sugerido a reclassificação do padrão sólido em carcinoma sólido simples, com marcação de pelo menos 60% de citoqueratina 8 (CK8), e p63 e actina de musculo liso- α (SMA) negativos; mioepitelioma maligno com mais de 90% das células marcando apenas p63/SMA; e o carcinoma bifásico com marcação dos dois tipos celulares, contendo pelo menos 60% positivas para CK8 (Yoshimura et al., 2014).

Mais recentemente outro estudo, comprovou que diferentes tipos celulares, células epiteliais, mioepiteliais ou ambas, podem estar proliferadas no tipo histológico chamado de “sólido” nos tumores mamários caninos. Com isso, foi feita uma nova proposta de divisão dos sólidos, baseado nas características histológicas e/ou imuno-histoquímicas, em carcinoma com padrão sólido, adenomioepitelioma maligno, mioepitelioma, maligno, carcinoma basalóide, carcinoma papilar sólido e carcinoma neuroendócrino (Nakagaki et al., 2022). Além de padrões histológicos e fenotípicos, foi identificado que há diferença no índice de proliferação celular, invasão linfática e metástase entre os tipos (Yoshimura et al., 2014; Nakagaki et al., 2022).

Os carcinomas sólidos da glândula mamária canina não são relatados como o tipo histológico mais comum nas casuísticas de neoplasias de cadelas (Santos et al., 2014; Nunes et al., 2018; Canadas et al., 2019; Rodrigues et al., 2023), porém apresentam grande importância, pois, em sua maioria, tem alta graduação histológica, invasão vascular e frequente metástase para linfonodos (Gamba et al., 2017; Zappulli et al., 2021). Adicionalmente, estão relacionados a um maior risco de morte associado ao câncer, quando se compara com carcinoma originado de tumor misto e outros tipos, como papilar e tubular (Nunes et al., 2018; Seung et al., 2021). Após a utilização da nova classificação das neoplasias de arranjo sólido, o adenomioepitelioma maligno e o carcinoma basaloide tem se mostrado os tipos mais frequentes dentre os sólidos (Nakagaki et al., 2022; Rodrigues et al., 2023).

O adenomioepitelioma maligno (ADMM) é uma neoplasia com duas populações celulares (epitélio e mioepitélio), no qual a malignidade pode se originar de um dos componentes celulares ou de ambos (Foschini et al., 2019; Nakagaki et al., 2023). É um tipo histológico raro nas mulheres, sendo mais visto em mulheres mais velhas, a partir de 60 anos (Foschini et al., 2019), entretanto, tem se mostrado comum nas cadelas (Yoshimura et al., 2014; Nakagaki et al., 2022). Histologicamente, as células se organizam em ninhos, o citoplasma tem moderada quantidade de vacúolos, apresentam pleomorfismo moderado, núcleo redondo com nucléolo evidente, e a atividade mitótica e o índice de proliferação celular são moderados (Nakagaki et al., 2022) (Figura 1A). Quando a transformação maligna é no componente epitelial, pode ocorrer aspecto de carcinoma invasivo. Já na parte mioepitelial, há maior quantidade de células alongadas e epitelioides com atipia nuclear e figuras de mitose frequentes (Foschini et al., 2019). Muitas vezes, a avaliação da neoplasia pela imuno-histoquímica é necessário para a distinção e identificação das células epiteliais e mioepiteliais. Nas cadelas, esse tipo apresenta marcação para pancitoqueratina (panCK) e proteína p63, sendo este marcador expresso em 10 a 90% das células (Nakagaki et al., 2022). No diagnóstico molecular, em mulheres, a marcação de RE pode ser positiva ou negativa (Foschini et al., 2019). Outra neoplasia de arranjo sólido que apresenta proliferação de células mioepiteliais é o mioepitelioma maligno (MM), em que mais de 90% das células exibem marcação nuclear para p63 (Nakagaki et al., 2022) ou SMA (Yoshimura et al., 2014). Nas cadelas é considerado um tipo raro. Histologicamente, as células são alongadas a redondas, com bordos indistintos e com citoplasma eventualmente vacuolizado, núcleos são ovalados e nucléolo frequentemente único e pequeno (Nakagaki et al., 2023) (Figura 1B).

O carcinoma basaloide (CB) é caracterizado por uma formação multinodular separado por moderado estroma fibroso (Nakagaki et al., 2023). A população celular forma arranjos

sólidos em que as células da periferia se organizam em paliçadas e apresentam um menor tamanho comparado às células mais centrais dos ninhos (Figura 1C). Frequentemente pode ocorrer metaplasia escamosa, e o índice de proliferação celular (Ki-67) é maior que a sua variante benigna (adenoma basalóide). As células neoplásicas marcam citoqueratina (CK) 14 nas células epiteliais da periferia, mas também pode ser positiva ao longo da proliferação. Cadelas que apresentam como neoplasias o CPS e o CB em estágio inicial não atingiram a mediana de sobrevida, enquanto as que desenvolvem o carcinoma com padrão sólido atingiram a mediada de sobrevida com 365 dias (Nakagaki et al., 2022).

O carcinoma papilar sólido (CPS) é caracterizado por uma proliferação exacerbada de papilas recobertas por múltiplas camadas de células epiteliais sobre um delicado estroma fibrovascular. As múltiplas camadas celulares se coalescem gerando o aspecto sólido da neoplasia (Figura 1D). As células podem ser redondas a alongadas, citoplasma eosinofílico, grande núcleo e nucléolo proeminente (Kulka et al., 2022; Nakagaki et al., 2022), e na mulher pode ter leve a moderada atipia nuclear (Mac Grogan et al., 2019). A coloração por Tricrômico de Gomori ou de Masson auxilia na visualização do estroma das papilas (Nakagaki et al., 2022). Em mulheres, esse tipo pode ser *in situ* ou invasor, e as células são forte e difusamente positivas para RE, todavia, o RP tem expressão variável, e a marcação de Ki-67 é geralmente baixa a intermediária (Mac Grogan et al., 2019), enquanto nas cadelas foi demonstrada ser alta (Nakagaki et al., 2022). Essa neoplasia é mais comum em mulheres pós-menopausa, principalmente durante os 70 anos de idade (Mac Grogan et al., 2019).

O carcinoma com padrão sólido (CSP) é pouco frequente em cadelas, entretanto, dentro os tipos de neoplasias sólidas é o que mostra maior índice de invasão vascular e metástase regional (Yoshimura et al., 2014; Nakagaki et al., 2023). Anteriormente tinha sido denominado de carcinoma sólido simples (Yoshimura et al., 2014). Microscopicamente, apresenta proliferação epitelial sólida, em que as células expressam predominantemente de pan CK, e menos de 10% expressam p63 (Nakagaki et al., 2022). Formam grande ninhos sólidos com escasso estroma, as células apresentam limites citoplasmáticos indistintos e citoplasma eosinofílico, núcleo redondo com nucléolos grandes e proeminentes. A atividade mitótica e o grau histológico são elevados (Nakagaki et al., 2022) (Figura 1E). Já os carcinomas neuroendócrinos da mama são frequentemente infiltrativos e, na histologia, apresentam arranjos sólidos de ninhos separados por fino septo fibrovascular. As células contêm fina granulação citoplasmática, a contagem mitótica é moderada a elevada (Nakahira et al., 2015; Nakagaki et al., 2020). A imunomarcação com cromogranina A positiva nas células epiteliais é importante

para o diagnóstico de neoplasia neuroendócrina, pois raramente a organização de rosetas é vista (Nakagaki et al., 2020) (Figura 1F).

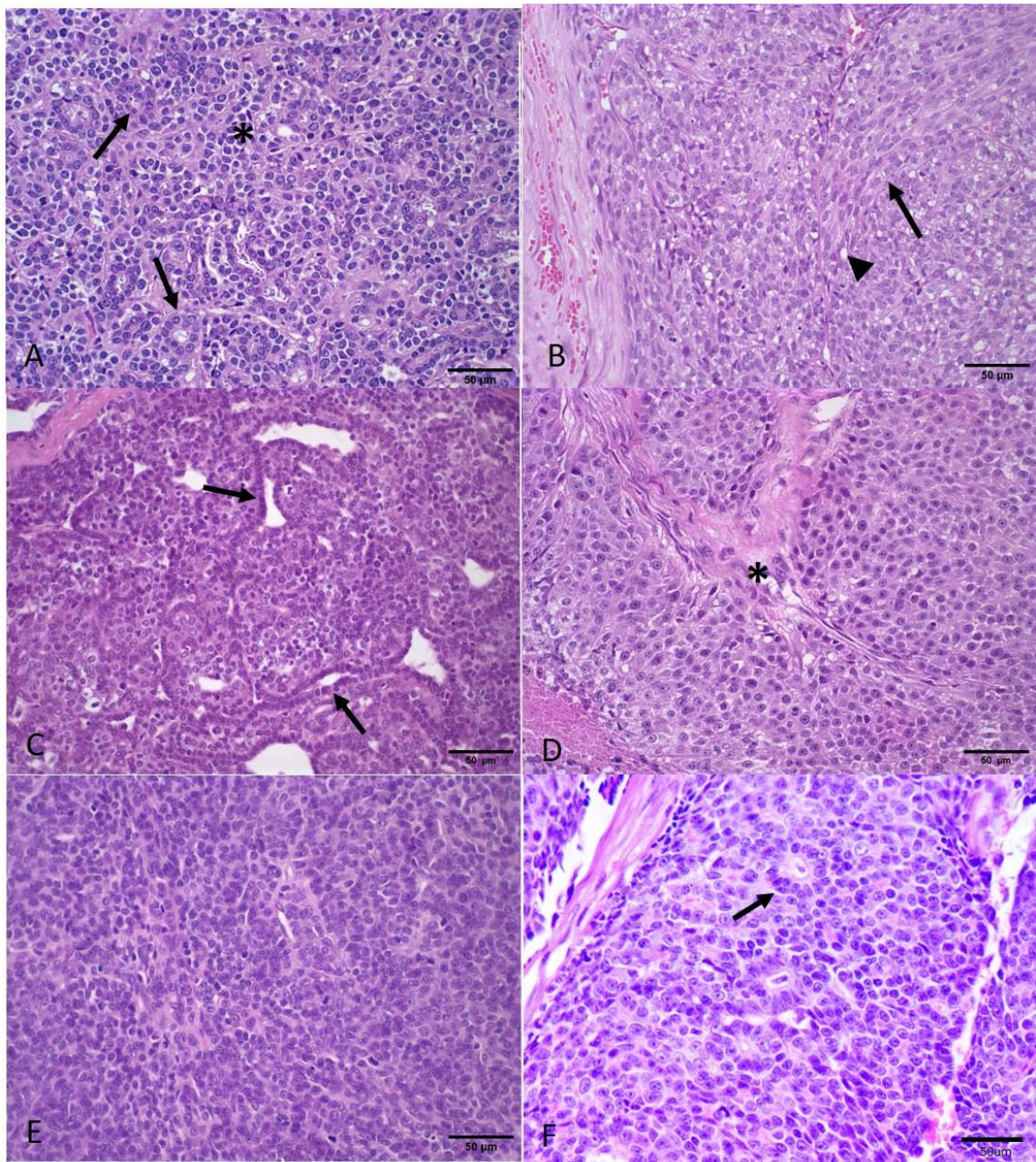


Figura 1. Microscopia das neoplasias de arranjo sólido da glândula mamária canina. A. Adenomioepitelioma maligno: células menores com núcleo redondo e hipercromático caracterizando as células mioepiteliais (*) e formações tubulares revestidas por células epiteliais (seta). B. Mioepitelioma maligno: células alongadas arranjadas em feixes (seta) e com vacúolos (cabeça da seta). C. Carcinoma basaloide: Lóbulos de células epiteliais apresentando as células periféricas menores e arranjas em paliçadas (seta). D. Carcinoma papilar sólido: múltiplas camadas de células epiteliais apoiadas em septo fibrovascular (*) que suporta uma papila. E. Carcinoma invasor padrão sólido: células epiteliais formando ninhos coalescentes. F. Carcinoma neuroendócrino: organização de rosetas HE, 400X. Barra=50µm.

2.3 Subtipos imunofenotípicos das neoplasias mamárias de cadelas, baseado na classificação humana

A grande variação genética dos tumores mamários humanos está associada a diferenças nos padrões morfológicos, fenotípicos e comportamentais, portanto a expressão gênica começou a ser estudada na medicina humana há algum tempo. Baseado na expressão gênica que codificam proteínas constituintes dos receptores hormonais (estrógeno e progesterona), de fator de crescimento epidermal humano (HER-2) e de citoqueratina basal (CK4 e 14) foram criados os subtipos intrínsecos do câncer de mama em mulheres, denominados luminal A, luminal B, HER-2 superexpresso, *basal-like*, e *normal like* (Perou et al., 2000; Sorlie et al., 2001). Porém, com o passar dos anos, outras propostas de divisão desses subtipos foram feitas, e outros subtipos foram criados, como luminal C, separando o *basal-like* e não *basal-like* (Parker, 2009). Essas classificações moleculares foram importantes para traçar fatores prognósticos e preditivos no câncer de mama em mulheres, entretanto, devido a análise molecular ser cara e pouco difundida, a imuno-histoquímica foi desenvolvida para esse fim. Com isso, foram classificados cinco imunofenótipos dos tumores de mama humano, o luminal A, luminal B HER-2 positivo, luminal B HER-2 negativo, HER-2 superexpresso e triplo-negativo (Goldhirsch et al., 2013). Nessa nova classificação a expressão do índice de proliferação celular, através da contagem de imunomarcação do Ki-67, subdividiu o Luminal A (Ki-67 baixo) do B (Ki-67 alto). Posteriormente, foi confirmado que as neoplasias dos dois subtipos apresentavam diferença na graduação histológica e isso refletiu em diferentes prognósticos e tratamento para as pacientes (Goldhirsch et al., 2013; Li et al., 2015). Assim, a inclusão da avaliação do índice de proliferação celular com base na contagem do Ki-67 foi importante para a separação de tumores que apresentam RE e/ou RP positivos e HER-2 negativo (Li et al., 2015). O mesmo pensamento também vem sendo desenvolvido na medicina veterinária, onde, por análise gênica e imuno-histoquímica, os subtipos têm sido diagnosticados tendo como base a literatura médica humana (Sassi et al., 2010; Im et al., 2014; Bergholtz et al., 2022; Nunes et al., 2022). Os subtipos das neoplasias mamárias das cadelas se baseiam na expressão de receptores hormonais, estrógeno (RE) e progesterona (RP), e na de marcadores de proliferação celular (Ki-67), que caracterizam a subdivisão Luminal (A e B) (Nunes et al., 2022), em fatores de crescimento epidermal humano (HER-2) e marcadores basais (citoqueratinas 14, p63) que determinam os tipos HER-2 superexpresso, *basal-like* e triplo-negativo, dependendo da classificação (Im et al., 2014; Varallo et al., 2019; Nunes et al., 2022) (Tabela 1).

TABELA 1. Classificação dos imunofenótipos utilizado para cadelas.

Autor	Tipo	Marcadores
Gama et al., 2008	Luminal A	RE positivo/HER-2 negativo
	Luminal B	RE positivo/HER-2 positivo
	HER-2 overexpressing	RE negativo/HER-2 positivo
	<i>Basal-like</i>	RE /HER-2 negativo e CK14 ou p63 positivo, ou E-caderina
	<i>Normal-like</i>	RE, RP, HER-2 Negativo
Im et al., 2014	Luminal A	RE positivo/HER-2 negativo
	Luminal B	RE positivo/HER-2 positivo
	HER-2 overexpressing	RE negativo/HER-2 positivo
	<i>basal-like</i>	RE /HER-2 negativo e CK14 ou p63 positivo
	<i>normal-like</i>	RE, RP, HER-2 Negativo
Varallo et al., 2019	Luminal A	RE e/ou RP -positivo, HER-2 negativo
	Luminal B	RE e/ou RP -positivo, HER-2 positivo
	HER2-overexpressing	RE e/ou RP-negativo HER-2 positivo
	Triplo-negativo	RE, RP, e HER2 negativo
Nunes et al., 2022	Luminal A	RE e/ou PR-positivo, HER-2 negativo e baixo Ki67 (<20%)
	Luminal B HER2-negativo	RE e/ou RP-positivo, HER-2 negativo e alto Ki67 ($\geq 20\%$)
	Luminal B HER2-positivo	RE e/ou RP-positivo e HER-2 positivo e qualquer Ki67
	HER2-overexpressing	RE e RP-negativo e HER-2 positivo
	Triplo-negativo	RE, RP, e HER-2 negativo

Levando em consideração esses subtipos, os tumores de imunofenótipo luminal A são os mais comuns nas neoplasias de cadelas, quando se considera a marcação de receptores hormonais (Gama et al., 2008; Im et al., 2014; Varallo et al., 2019), porém no trabalho de Nunes et al. (2022) quando o Ki-67 é mensurado, a maioria das neoplasias são classificadas como

luminal B, em que o Ki-67 é igual ou maior que 20%. Os tipos luminais também são os que apresentam melhor prognóstico, e as pacientes (mulheres e cadelas) têm maior tempo de sobrevida e menor índice de metástase (Cheang et al., 2009; Prat et al., 2015, Abadie et al. 2018; Varallo et al., 2019). Cadelas com tumores *basal-like* (Im et al., 2014) e triplo negativo apresentam significativamente maior tamanho, maior índice de Ki-67 e menor tempo de sobrevida (Abadie et al., 2018; Pastor et al., 2020; Nunes et al., 2022).

2.4 Infiltrado inflamatório no câncer de mama

A ideia de que as células cancerosas interagem com o ambiente no qual o tumor está inserido, e que as células imunes podem reprimir o crescimento tumoral foi primeiro proposto por Paul Ehrlich em 1909. Muitos anos depois, foi proposta a teoria da imunovigilância no câncer, suspeitando-se que linfócitos agiam como “guardas” para identificar e matar células que sofreram mutações (Burnet, 1957; Burnet, 1970), porém, essa teoria ficou muito tempo sem comprovação. Com o passar dos anos, e a partir dos estudos de imunologia baseados em camundongos e humanos, fortes indícios deram suporte à existência de um sistema de imunovigilância do câncer e, permitiram o desenvolvimento de novas pesquisas (Dun et al., 2002). Assim, foi possível comprovar que a inflamação pode ter um papel pró ou antitumoral dependendo das células imunes presentes, dos tipos de citocinas, quimiocinas, enzimas e fatores de crescimento que estão sendo produzidos, e da característica e localização da inflamação dentro do tumor (Nguyen et al., 2018; Jakubowska et al., 2017; Shrihari, 2017). As células inflamatórias liberam mediadores que ativam mecanismos intracelulares para proliferação, instabilidade genômica e transformação maligna, além de gerar alterações da matriz extracelular e seus componentes (Felix et al, 2016). Um fator que afeta diretamente a inflamação nas neoplasias mamárias é o subtipo molecular ou imunofenótipo (Adams et al, 2015).

A caracterização do microambiente tumoral com a avaliação de leucócitos infiltrantes já vem sendo realizada em alguns tipos de neoplasias. Em humanos, o estudo da inflamação é feito no carcinoma colorretal (Klintrup et al., 2005; Mlecnik et al., 2011; Jakubowska et al., 2017; Galdiero et al, 2019), carcinoma de células escamosas oral e cutâneo (Shimizu, 2018; Stravodimou et al., 2018); carcinomas renal, de cabeça e pescoço, hepatocelular e ducto-pancreático (Shen et al., 2014); carcinomas mamários (Zhu et al., 2015; Denkert et al., 2018; Koletsa et al., 2020) e carcinoma de células de transicionais de bexiga (Liu et al., 2018).

Na veterinária, os linfócitos ainda são as células mais estudadas no microambiente tumoral canino, como por exemplo em melanomas oral e cutâneos (Gregório et al., 2016;

Porcellato et al., 2019), carcinoma de células de transicionais de bexiga (Inoue et al., 2017; Maeda et al., 2019) e no próprio câncer de mama (Estrela-Lima et al., 2010; Souza et al, 2018; Nelson et al, 2021; Muscatello et al. 2022).

As neoplasias em estágios iniciais apresentam maior quantidade de células Natural Killer (NK) CD16⁺ e linfócitos TCD8⁺ que assumem uma função importante de reconhecer e eliminar as células neoplásicas (Zabotina et al., 2018). As células NK reconhecem células neoplásicas que não expressam moléculas do complexo de histocompatibilidade (MHC) de classe I, além disso, ativam outros receptores que auxiliam na destruição da neoplasia, como o receptor de natural killer D2 (NKD2). Os linfócitos TCD8⁺ são ativados a partir do reconhecimento de moléculas associadas ao MHC I nas células apresentadoras de antígeno (CAA), principalmente células dendríticas. Dessa forma, há uma ativação e expansão clonal dos linfócitos, assim como das células NK, que ao reconhecerem as células tumorais tentam induzir a sua morte por liberação de perforinas e granzimas, e por apoptose via a ligação da molécula Fas em sua superfície, com o seu ligante (FasL) na célula neoplásica. Ao mesmo tempo, as células neoplásicas danificadas liberam moléculas associadas a danos (DAMP's) que são reconhecidas por células do sistema imune inato (CAA, macrófagos, neutrófilos). Essas células da imunidade inata também reconhecem diferenças na constituição das membranas celulares e expressão de antígenos tumorais. Assim, vários tipos de citocinas são liberadas, como o interferon γ (INF- γ) que favorecem uma a diferenciação de linfócitos imaturos a linfócitos TCD4⁺ helper 1 (h1). Esses, aumentam a secreção de INF- γ e α , amplificando a ativação de macrófagos, neutrófilos e suas diferenciações para macrófagos M1 e neutrófilos N1 caracterizando uma resposta anti-tumoral (Amens et al, 2021; Nelson et al, 2021; Estrela-lima e Lopes, 2023). Além disso, nessa fase inicial, os linfócitos B podem assumir função anti-tumoral, principalmente quando se maturam em linfócitos B de memória. Assim, estão envolvidos no processamento e na apresentação de antígenos (Hu et al., 2021) e participam da ativação de linfócitos citotóxicos (Lei et al., 2020; Garaud et al., 2019).

Como a progressão da neoplasia, as células neoplásicas iniciam uma resposta reguladora e imunossupressora, principalmente através da secreção do fator de transformação de crescimento β (TGF- β), interleucina (IL) 10, IL-17 e pela troca de receptores de superfície celular (Lei et al., 2020; Garaud et al., 2019; Kwantwi et al., 2021; Estrela-lima e Lopes, 2023), além de aumento da secreção de Fator Estimulador de Colônias de Granulócitos e Macrófagos (CSF-GM) (Casbon et al., 2015). Assim, há aumento da quantidade de linfócitos TCD4⁺ helper 2 (h2) e diferenciação de linfócitos reguladores (principalmente que expressam Foxp3), aumento da quimiotaxia de células supressoras mielóides, macrófagos e neutrófilos, que se

diferenciam para M2 e N2. Todas as células favorecem a secreção de citocinas angiogênicas, que aumenta a expressão de genes associado a transição epitélio-mesênquima, e reduzem a resposta anti-tumoral celular (Zabotina et al., 2018; Sadeghalvad et al., 2020; Amens et al., 2021; Damasceno et al., 2023) (Figura 2). Também tem sido estudada a diferenciação de linfócitos B reguladores e produção de citocinas que aumentam a angiogênese e recrutam células derivadas mieloides, favorecendo a apresentação de antígenos. Entretanto, essas funções ainda precisam ser melhor compreendidas (Lei et al., 2020; Garaud et al., 2019). Em camundongos, observa-se aumento de linfócitos B favorecendo a progressão neoplásica e formação de nicho pré-metastático em linfonodos que drenam a região da neoplasia mamária (Gu et al., 2019).

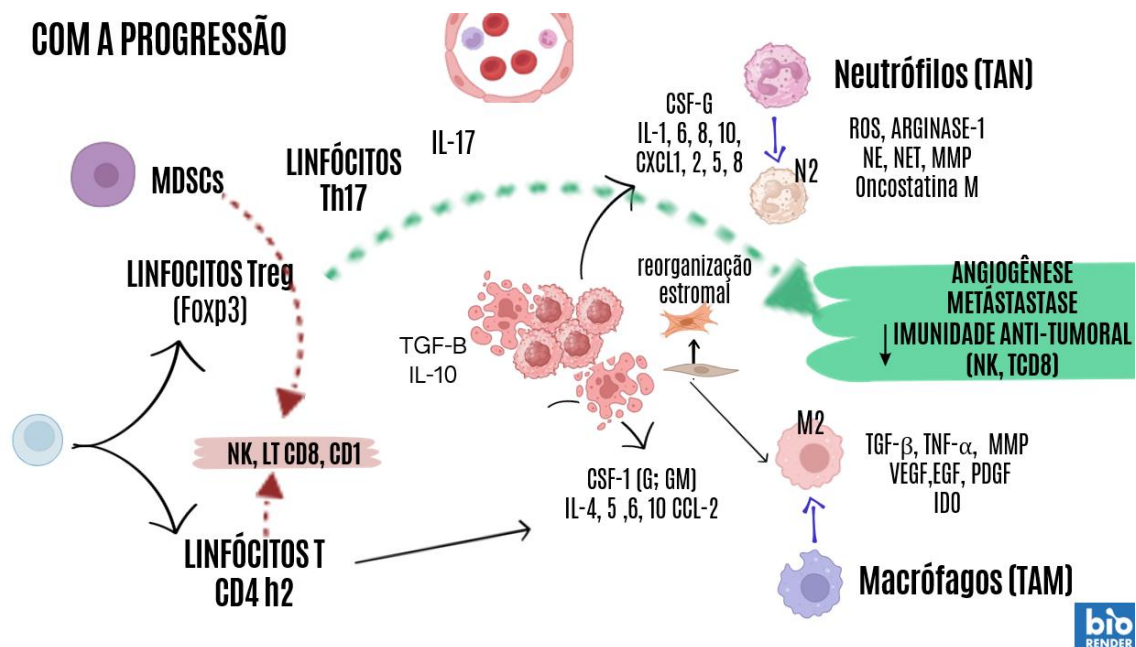


Figura 2. Resposta inflamatória pró-tumoral. Em um microambiente rico em TGFβ e IL-10, induzido pela neoplasia, os linfócitos se diferenciam TCD4⁺ h2, linfócitos T reguladores que além de suprimir as células responsáveis pela imunidade antitumoral secretam citocinas e quimiocinas que favorecem a quimiotaxia e diferenciação de células derivadas mieloides, macrófagos e neutrófilos N2, linfócitos Th17, e consequentemente induzem a reorganização da matriz extracelular, angiogênese e metástase. MDSCs: células supressoras mieloides; NK: natural Killer; ROS: espécies reativas de oxigênio; MMP: metaloproteases; VEGF: fator de crescimento endotelial, EGF: fator de crescimento epitelial; PDGF: fator de crescimento de fibroblastos; IDO: indoleamina 2,3-dioxigenase; CSF-G: fator estimulador de granulócitos; CSF-GM: fator estimulador de macrófagos e granulócitos.

2.4.1 Linfócitos Infiltrantes do tumor (TILs) no câncer de mama

Linfócitos são importantes no microambiente tumoral e podem afetar o equilíbrio da resposta imune e tolerância, participar do desenvolvimento e progressão tumoral e podem

contribuir para angiogênese e invasividade (Carvalho et al., 2014; Huang et al., 2015; Yu et al., 2016; Zabolina et al., 2018). Esses linfócitos que infiltram o tumor são denominados linfócitos infiltrantes tumorais, Tumour-infiltrating lymphocytes (TILs), e os principais subtipos no microambiente tumoral são os linfócitos T citotóxicos (CD8⁺), T helper (CD4⁺), B (CD79⁺ ou CD20⁺) e T reguladores (Amens et al., 2021).

Há alguns anos a avaliação do infiltrado linfocitário no câncer de mama vem sendo feito na medicina humana, inicialmente sendo usado apenas a avaliação da densidade na lâmina corada em hematoxilina e eosina (H&E) (Aaltomaa et al., 1992), e com o tempo, a análise de imuno-histoquímica foi introduzida nessa avaliação dos TILs para definir os subtipos de linfócitos predominantes nas neoplasias. Os TILs e os subtipos de linfócitos são associados a características morfológicas (grau, tamanho, pleomorfismo, contagem mitótica) e imunofenótipos para estabelecimento de fatores prognósticos e preditivos (Denkert et al., 2010, Mahmoud et al., 2011, Mohammed et al., 2013, Gao et al., 2020). Com o crescente estudo dos TILs em câncer de mama de mulheres, foi criado em 2013 um grupo com pesquisadores para discutir a importância, bem como metodologias para a avaliação da infiltração dessas células. Nas mulheres a avaliação dos TILs estromal nas lâminas de H&E é feita pela porcentagem de ocupação, pelos linfócitos e plasmócitos, da área estromal entre os ninhos de células neoplásicas. Acredita-se que esses TILs estromal tenham melhor reprodutibilidade, pois é mais frequente e mais fácil de avaliar que o intratumoral (Salgado et al., 2015).

A maior intensidade e a quantidade de TILs no microambiente tumoral estão associadas a receptores de estrogênio e progesterona negativos e maior escore de expressão de HER-2, maior grau tumoral e maior índice de Ki-67 (Lee et al., 2015; Wang et al., 2016; Koletsa et al., 2020).

O imunofenótipo do câncer de mama influencia a quantidade e a composição do infiltrado inflamatório do microambiente (Glajcar et al., 2019; Zhu et al., 2019). Neoplasias do subtipo luminal apresentam menor intensidade de TILs estromal e intratumoral quando comparado a tumores mais agressivos, como os tumores TN e HER-2 (Adams et al., 2015, Solinas et al., 2017). Esses apresentam maior intensidade de TILs, que pode ser explicado pela alta taxa proliferativa e capacidade mutagênica, gerando maior antigenicidade (Adams et al., 2015; Glajcar et al., 2019).

Em tumores TNs, a quantidade de linfócitos TCD8⁺ é considerado um fator prognóstico, pois quanto maior a infiltração desses linfócitos na periferia do tumor, maior a sobrevida, e a menor quantidade desses linfócitos aumenta duas vezes o risco de morte pelo câncer

(Vihervuori et al., 2019). Já nas neoplasias luminais, a maior infiltração de linfócitos TCD8⁺ está associada a pior tempo de sobrevida (Sobral-leite et al., 2019).

Denkert et al. (2010) mostraram que neoplasias com maior quantidade de linfócitos no tumor, apresentaram uma melhor resposta ao tratamento quimioterápico, sendo o TILs intratumoral considerado um fator preditivo independente. Esses autores sugeriram que as destruições das células tumorais pela quimioterapia favorecem a liberação de antígenos tumorais, e desencadeiam uma resposta imune contra as neoplasias, em pacientes previamente sensibilizados. Após isso, outros estudos também têm demonstrado que a alta densidade de TILs estromal exerce um efeito prognóstico e preditivo positivo, em pacientes com neoplasias TN e HER-2 positivo (Adams et al., 2015; Lee et al., 2015; Vihervuori et al., 2019, Gao et al., 2020). Segundo Koletsa et al. (2020), uma maior quantidade de linfócitos CD3⁺ total, nas neoplasias luminais B, aumentam o tempo de sobrevida e reduz o risco de óbito, quando associado ao tratamento quimioterápico.

Os linfócitos B também constituem os TILs, porém estão presentes em menor quantidade quando comparado a população de linfócitos T (Hu et al, 2021). Assim, como é visto em relação aos linfócitos T, a quantidade da infiltração de linfócitos B é proporcional ao índice de Ki-67, grau histológico, e inversamente proporcional aos receptores hormonais. A presença de linfócitos B é significativamente associada a melhor prognóstico para neoplasia TN e HER-2, com pacientes apresentando maior intervalo livre de doença e sobrevida (Garaud et al., 2019; Sadeghalvad et al., 2020; Kuroda et al., 2021). Mas em neoplasias invasivas, linfócitos B do tipo regulador podem ser encontrados em maior quantidade exercendo um efeito pró-tumoral. Isso foi demonstrado em mulheres com carcinoma ductal invasivo, em que linfócitos B reguladores (CD45⁺) formavam maior quantidade de agregados na área peritumoral quando comparado a carcinoma ductal *in situ*, e essa característica foi diretamente associados a maior frequência de metástase nodal (Ishigami et al., 2018). Em modelos experimentais de neoplasias mamárias, esses linfócitos B reguladores secretam grande quantidade de IL-10, diminuindo a ação de linfócitos T anti-tumorais. (Gu et al., 2019).

Nas cadelas, os linfócitos são as células que predominam no microambiente tumoral de mama, mas também podem ser vistos plasmócitos, macrófagos, neutrófilos e eosinófilos (Estrela-Lima et al., 2010; Lopes-neto et al., 2017; Souza et al., 2018). Diversas metodologias vêm sendo usadas para análise morfológica a fim de quantificar o infiltrado inflamatório nas neoplasias mamárias de cadelas, e associar a fatores prognósticos. Dentre as metodologias estão o uso de um sistema de gradação da inflamação, nas lâminas coradas em H&E, usando a intensidade e distribuição da inflamação (Estrela-Lima et al., 2010; Kim et al., 2013), a

contagem dos tipos de linfócitos em campos *Hot spot* para estabelecer um ponto de corte (Estrela-Lima et al., 2010), ou ainda a análise da porcentagem de TILs estromal seguindo a metodologia usada em mulheres (Muscatello et al., 2022).

Os estudos demonstram que a infiltração de linfócitos foi significativamente maior em carcinomas mamários com invasão linfática e com alto grau histológico (Kim et al., 2013; Lopes-Neto et al., 2017), e que cadelas que apresentam maior infiltração de linfócitos tumoral (>600 linfócitos em 8 campos em objetiva de 100x) apresentam menor tempo de sobrevida (Estrela-Lima et al., 2010). Os tumores malignos tendem a apresentar maior infiltração de linfócitos T CD3⁺ que os benignos (Carvalho et al., 2011; Saeki et al., 2012), e tipos histológicos mais agressivos como carcinossarcoma e carcinoma sólido apresentam maior infiltração de linfócitos T CD3⁺ quando comparados a tipos considerados menos agressivos com carcinoma túbulo-papilar (Carvalho et al., 2011).

Comparando a infiltração tumoral de linfócitos T e B, cadelas com carcinoma em tumor misto que apresentavam metástase para linfonodo tiveram mais infiltração de linfócitos B CD79⁺ tumoral (cutt off $\geq 66.7\%$), já os carcinomas em tumor misto que não apresentam metástase tinham significativamente maior infiltração de linfócitos T CD3⁺. E dentro dos subtipos de linfócitos T, há uma maior porcentagem de TCD4⁺ em cadelas com metástase, já o LTCD8⁺ predomina em cadelas sem metástase para linfonodo (Estrela-Lima et al., 2010).

Nas cadelas, pouco se sabe a relação entre a inflamação e os imunofenótipos. Kim et al. (2013), não encontraram diferença de TIL entre os imunofenótipos, e Abadie et al., (2018) observaram que a presença de inflamação peritumoral aumenta o risco de óbito por neoplasias em 1,54 vezes em neoplasias triplo-negativas.

2.4.2 Neutrófilos infiltrantes do tumor (TANs) no câncer de mama

As próprias células neoplásicas e os TILs produzem quimiocinas, citocinas e fator estimulador de colônias de granulócitos (CSF-G) que aumentam a migração de neutrófilos para o microambiente tumoral, como a CCL20 e IL-17 (Benevides et al., 2015; Casbon et al., 2015; Timaxian et al., 2021). Os neutrófilos no contexto de inflamação associada ao mecanismo tumoral são denominados de neutrófilos infiltrantes do tumor (TANs), e podem ter diferentes estágios de ativação. Em ambientes ricos de TGF- β adquirem o fenótipo pró-tumoral (N2), já em locais com baixo TGF- β apresentam o fenótipo anti-tumoral (N1) (Fridlender et al., 2009). O efeito anti-tumoral está ligado a secreção de mediadores citotóxicos como TNF- α , IL-1 β e IFN, espécies reativas de oxigênio (ROS), maior expressão de Fas-L, que induzem a apoptose das células tumorais e ativação de linfócitos TCD8⁺. Já o efeito pró-tumoral se deve a produção

de fatores angiogênicos como fator de crescimento endotelial (VEGF), arginase, proteases como melatoproteases (MMP-9) (Fridlender et al., 2009), e indução de moléculas, como PDL-1, que reduzem a ativação de linfócitos T com função anti-tumoral (Kwantwia et al., 2021). A produção de oncostatina M por neutrófilos em contato com células de tumores de mama, induzem a secreção de VEGF que favorece a angiogênese, invasividade e metástase (Queen et al. 2005).

Em modelos *in vitro* e *in vivo* foi possível identificar que a depender do tipo de citocina no microambiente ou dos receptores presentes, os neutrófilos podem estar associados a melhor ou pior sobrevida (Queen et al. 2005; Timaxian et al., 2021). Foi observado que neoplasias com RE positivos, em estágios avançados, apresentam aumento da quantidade de TGF- β no microambiente, favorecendo a quimiotaxia e polarização de neutrófilos. Além disso, *in vitro*, os neutrófilos migram junto com as células neoplásicas para dentro de vasos sanguíneos, auxiliando na disseminação e no nicho pré-metastático (Vazquez-Rodriguez et al., 2017)

Os trabalhos científicos da associação de neutrófilos com características morfológicas das neoplasias mamárias, e fatores prognósticos e preditivos são escassos na medicina humana. Em mulheres a quantidade média de neutrófilos é maior em tumores TN e HER-2 positivo e, muito pequena nas neoplasias positivas para receptores hormonais (Perez-de-Celis et al., 2017). A alta densidade de TANs está associada à menor tempo livre de doença (Kwantwia et al., 2021). Um estudo em cadelas, a quantidade de neutrófilos por unidade de área foi maior em carcinoma em tumor misto e carcinoma tubular, em comparação ao carcinoma sólido e tumor misto benigno (Souza et al., 2018), porém não se tem informações sobre associação dessas células com aspectos morfológicos e sobrevida nas neoplasias mamárias.

Portanto, as neoplasias de arranjos sólidos apresentam características histológicas e fenotípicas distintas, assim como prováveis diferenças comportamentais. Sabe-se, que as neoplasias mamárias de cadelas são predominantemente luminais, e raramente, HER-2 superexpresso, mas não temos estudos dos imunofenótipos nos subtipos de sólidos, e se esses imunofenótipos contribuem para o prognóstico desfavorável desses tipos histológicos. Ademais, em mulheres já é comprovado que os TILs e a sua distribuição espacial associado a tipos histológicos e subtipos moleculares, tem valor como fator prognóstico e preditivo, porém isso é pouco estudado nas cadelas.

3. HIPÓTESES

O infiltrado inflamatório é maior em neoplasias de arranjos sólidos com maior atividade proliferativa (contagem mitótica e marcação de Ki-67), com presença de metástase e com imunofenótipos de pior prognóstico.

Neoplasias com infiltrado inflamatório rico em linfócitos T CD3⁺ apresentam melhor prognóstico e estão associadas a maior sobrevida das cadelas, que as que apresentam mais intensidade de linfócitos B. Já os neutrófilos são encontrados em menor quantidade no microambiente, mas aumentam em neoplasias de com piores características histológicas (anisocariose e pleomorfismo acentuada, com contagem mitótica elevada) e metástase.

4. OBJETIVOS

4.1 Objetivo geral

Caracterizar infiltrado inflamatório peri e intratumoral nas neoplasias mamárias de arranjo sólido da glândula mamária canina, com ênfase no infiltrado linfocitário e neutrofílico e correlacionar com imunofenótipos, fatores prognóstico (tamanho tumoral, metástase para linfonodo, contagem mitótica, índice de proliferação de Ki-67), expressão de ciclo-oxigenase (Cox-2), necrose e sobrevida das cadelas.

4.2 Objetivos específicos

Identificar os imunofenótipos das neoplasias de arranjo sólido da glândula mamária canina.

Caracterizar e quantificar o grau de inflamação peri e intratumoral nos tipos de neoplasias de arranjo sólido da glândula mamária canina através da avaliação histopatológica.

Quantificar a infiltração de neutrófilos e linfócitos nas lâminas histológicas, e linfócitos T e B, pela imuno-histoquímica, nas áreas peri e intratumoral.

Correlacionar os imunofenótipos, o grau de inflamação, a intensidade de TILs, TANs, linfócitos T e B entre si, e com os tipos histológicos de neoplasias sólidas, contagem mitótica, tamanho tumoral, metástase para linfonodo, índice de proliferação de Ki-67 e Cox-2, e a sobrevida global e específica das cadelas.

5. MATERIAL E MÉTODOS

5.1 Obtenção e seleção das amostras

Esse foi um estudo retrospectivo em que foram avaliadas 120 casos de neoplasias classificadas como carcinoma sólido ou carcinoma basaloide de cadelas obtidas durante os anos

de 2011 a 2022. Dessas, foram selecionadas 65 amostras de neoplasias de arranjo sólido da glândula mamária. As amostras (lâminas histológicas coradas em Hematoxilina e eosina e blocos de parafina) foram provenientes do acervo do Laboratório de Patologia comparada do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais (UFMG), do Laboratório de Patologia da Universidade Vila Velha (UVV), do laboratório de patologia Celulavet e do Centro de Pesquisa de Oncologia Mamária da Universidade Federal da Bahia (UFBA).

Crítérios de seleção

Foram selecionadas neoplasias com diagnóstico de “carcinoma sólido” ou “carcinoma basaloide” da glândula mamária de cadelas de acordo com os critérios adotados pela Organização mundial de Saúde (OMS) (Misdorp et al., 1999) e pelo Consensus for the Diagnosis, Prognosis and Treatment of Canine Mammary Tumors (Cassali et al., 2020).

Crítérios de inclusão

As amostras usadas na pesquisa deveriam ser oriundas de cadelas com informações de idade, localização da neoplasia, tamanho tumoral, informação de status de linfonodo regional. Além das lâminas coradas em Hematoxilina e Eosina (H&E), as amostras selecionadas deveriam estar em blocos de parafina.

Para o estudo de sobrevida o critério de inclusão foi ter informações da data de mastectomia para o cálculo da sobrevida. Cadelas que apresentavam mais de uma neoplasia caracterizada como sólida, foi selecionada a neoplasia que apresentava maior contagem mitótica e maior índice de Ki-67.

Crítérios de exclusão

Amostras que estavam insuficientes nos blocos de parafina para realização de cortes de imuno-histoquímica para determinar perfil imunofenotípico e inflamatório. E cadelas com aparecimento de outras neoplasias malignas de origem não-mamária (ex. carcinomas de outras regiões, mastocitoma, hemangiossarcoma, melanoma), ou neoplasias de mama de pior prognóstico (carcinossarcoma, carcinoma micropapilar, carcinoma lobular pleomórfico) segundo Cassali et al. (2020).

5.2 Diagnóstico e graduação histopatológica

O diagnóstico histopatológico foi feito para todos os casos por dois patologistas (GDC e MCF), e as neoplasias da glândula mamária foram classificados como carcinomas sólidos de acordo com os critérios adotados pela Organização mundial de Saúde (OMS) (Misdorp et al., 1999) e pelo Consensus for the Diagnosis, Prognosis and Treatment of Canine Mammary Tumors (Cassali et al., 2020). Posteriormente, essas neoplasias foram caracterizadas através de análise morfológica e imuno-histoquímica nos subtipos ADMM, CB, CPS, CSP, MM e carcinoma neuroendócrino de acordo com Nakagaki et al, 2022. Os linfonodos regionais, também foram avaliados, para a detecção de células neoplásicas. As neoplasias caracterizadas como carcinoma neuroendócrino não foram usadas, devido a baixa frequência desse tipo nas amostras. Durante a avaliação histopatológica, os tumores também foram graduados de acordo com o Sistema de Nottingham (Elston & Ellis, 1991), com exceção dos ADMM e MM. O ADMM pode apresentar áreas epiteliais benignas, e pode ser difícil avaliar sua área de invasão epitelial, e o MM é composto por mais de 90% de tecido mioepitelial, com pouco epitélio. Os dados clínicos dos casos selecionados foram obtidos pela ficha clínica ou fornecidos pelo laboratório de origem da amostra. Como as informações sobre avaliação de metástase a distância não estavam completas, não foi determinado o estadiamento clínico tumoral (TNM) do paciente, sendo usando apenas o critério para determinação de tamanho (T) e metástase nodal (N) segundo Organização Mundial da Saúde (OMS) (Owen, 1980). O tamanho tumoral foi estratificado em T1 (<3cm), T2 (entre 3 e 5 cm) e T3 (>5cm), seguindo o sistema TNM (Owen, 1980).

5.3 Realização da técnica de Imuno-histoquímica para definição dos imunofenótipos, marcação de Cox-2 e linfócitos CD3⁺ e CD79⁺

Para as reações imuno-histoquímicas, secções de 4 µm de espessura foram obtidas a partir dos blocos de parafina e montadas em lâminas de vidro histológicas gelatinizadas. Os cortes foram desparafinizados e reidratados, e subsequentemente submetidos à recuperação antigênica por calor (125°C/20minutos) em uma câmara de pressão úmida (*Pascal Pressure Chamber*®, Dako®, Carpinteria, CA, Estados Unidos) com solução de citrato (pH 6,0). O bloqueio de peroxidase endógena e de proteínas não específicas foram realizadas com solução comercial do sistema Novolink® (Leica Biosystems Newcastle Ltd., UK) de acordo com a recomendação do fabricante, seguido da incubação dos tecidos com os anticorpos primários (Tabela 2). O anticorpo secundário utilizado também era do sistema Novolink e a imunorreatividade foi

visualizada com o cromógeno 3'-diaminobenzidina (DAB do sistema Novolink®) por 3 minutos, e contra-corado com Hematoxilina.

A imuno-histoquímica para receptor de estrógeno (RE) e progesterona (RP), fator de proliferação de celular Ki-67 e Fator de crescimento epidermal humano (HER-2) foram feitas em todas as amostras selecionadas para a determinação do imunofenótipo (tabela 2). As avaliações foram realizadas utilizando microscópio Olympus BX-40 com câmera digital *Spot Insight Color* e com auxílio do software de captura SPOT→versão 3.4.5. Para a avaliação da marcação de RE e RP foi utilizado um sistema de escore, sendo observado marcação nuclear em qualquer intensidade (negativo: <1%, +: 1-25% de células marcadas; ++: 26-50% de células marcadas, +++: 51-75%; ++++: >75% de células marcadas) (Hammond et al., 2010). Para a análise do Ki-67 foi contabilizado a marcação nuclear em 500 células, no sistema de imagem Image J®, e foi considerado o ponto de corte de $\geq 20\%$, para alto índice de marcação. A expressão do HER-2 seguiu as diretrizes do escore realizado por Wolff et al. (2013), e foi considerado o escore 0, 1+ e 2+ negativos, e 3+ positivo. A partir daí as neoplasias foram categorizadas em luminal A (RE e/ou RP-positivo, HER2-negativo e Ki67 <20%); luminal B HER-2 negativo (RE e/ou RP-positivo, HER2-negativo e Ki67 $\geq 20\%$), luminal B HER-2 positivo (RE e/ou RP-positivo e HER2-positivo e qualquer Ki67), HER-2 superexpresso (RE e RP-negativo e HER2-positivo); Triplo negativo (RE, RP, e HER2 negativo), seguindo as diretrizes do consenso St Gallen (Goldhirsch et al., 2013) e adotado por Nunes et al. (2022).

Tabela 2. Especificações dos anticorpos utilizados na pesquisa.

Anticorpo	Clone	Diluição	Fabricante	Incubação
RE	Ep1	Pronto para uso	Dako	<i>overnight</i>
RP	hpra2	1:50	Thermo Fischer	<i>overnight</i>
HER-2	c erb 2	1:200	Thermo Fischer	<i>overnight</i>
Ki-67	mib 1	1:50	Dako	<i>overnight</i>
Cox-2	Sp21	Pronto para uso	Invitrogen	<i>overnight</i>
CD3	A052	1:200	Dako	<i>overnight</i>
CD79	HM47	1:400	Biolegend	<i>overnight</i>

A avaliação de Cox-2 baseou-se na marcação citoplasmática de células tumorais com a realização de escore de distribuição (0= ausente, 1= menos que 10% de células marcadas, 2= 10 a 30% células marcadas, 3= 31 a 60% de células marcadas, 4= maior que 61% de células marcadas) e intensidade (0= ausente, 1= marcação fraca (+), 2= marcação moderada (++) e 3=

marcação forte (+++). Para estabelecer o escore final, multiplica-se a distribuição por intensidade (Lavale et al., 2009).

A marcação positiva para linfócitos CD3⁺ e CD79⁺ foi considerada apenas a marcação citoplasmática, difusa ou granular. Como controle positivo foi utilizado linfonodos normais de cães, e para o controle negativo os anticorpos primários foram substituídos por solução salina tamponada em fosfato (PBS).

5.4 Graduação do infiltrado inflamatório nas lâminas histológicas

A graduação do infiltrado inflamatório (peritumoral e intratumoral) foi feita através da avaliação de uma lâmina de cada caso (quando a neoplasia tinha mais de uma amostra) contendo a área na borda e do centro da neoplasia, coradas em H&E. No caso de neoplasia com mais de 2cm, a lâmina com fragmento de aproximadamente 2 cm, que representasse parte peri e intratumoral, com melhor representatividade da neoplasia foi escolhida (características celulares melhores preservadas, menor necrose, hemorragia ou artefato, e como presença de inflamação). A avaliação foi realizada nas objetivas de 10x, seguida da objetiva de 20x, utilizando o microscópio Olympus BX-40. Foi considerada inflamação peritumoral, a presença de células mononucleares no tecido conjuntivo da cápsula ou da margem histológica (quando não havia cápsula) ao redor do tumor e sem contato direto com a neoplasia; e intratumoral, quando se localizavam no tecido conjuntivo fibroso entre os ninhos neoplásicos e dentro dos ninhos, exceto os que estavam na área de necrose. A graduação da inflamação foi feita separadamente da área peri e intratumoral, através de um escore de distribuição (focal exibindo de 1 a 3 focos de infiltrado inflamatório – 1, multifocal com mais de 3 focos – 2, difusa com distribuição da inflamação em toda a área tumoral – 3) e intensidade (discreta – 1, moderada – 2, intensa – 3). A partir daí a distribuição foi multiplicada pela intensidade resultando na graduação da inflamação (Grau I – escores entre 1 a 3; Grau II – escores entre 4 a 6; Grau III – escores entre 7 a 9) (Estrela-Lima et al., 2010; Kim et al., 2013).

5.5 Caracterização e quantificação do infiltrado de linfócitos e neutrófilos nas lâminas histológicas e na imuno-histoquímica

Foram capturadas imagens de oito campos *hot spots*, da área peritumoral e da área intratumoral, em 600x (objetiva 60x e ocular 10x) das lâminas coradas em H&E (as mesmas usadas para a graduação da inflamação), contendo um fragmento de cada neoplasia para quantificação dos TILs e TANs da área peritumoral e da área intratumoral, separadamente. Para a quantificação de linfócitos marcados para CD3⁺ e CD79⁺ também foram capturadas imagens

de oito campos em 400x, seguindo a mesma metodologia de captura feita nas lâminas coradas em H&E. As fotomicrografias foram feitas usando câmera digital Spot Insight Color adaptada no microscópio Olympus BX-40 com auxílio do software de captura SPOT-versão 3.4.5. (Estrela-Lima et al. 2010).

A identificação das células inflamatórias (neutrófilos e linfócitos) nas lâminas histológicas foi baseada no aspecto morfológico de cada célula. As células inflamatórias (linfócitos e neutrófilos) e os linfócitos CD3⁺ e CD79⁺ foram contadas separadamente na área peritumoral e intratumoral nos oito campos fotografados para cada área. Por fim, o número de cada tipo de célula inflamatória, nas áreas peritumoral e intratumoral, foi somado separadamente para cada neoplasia. Após a quantificação final de cada neoplasia, a mediana para cada tipo celular, na histologia e na imuno-histoquímica, foi estabelecida como ponto de corte para caracterizar como alta e baixa intensidade de infiltrado inflamatório.

5.6 Acompanhamento dos pacientes

As informações foram obtidas por meio de contato telefônico com os veterinários responsáveis pelo caso ou com os tutores. A sobrevida foi determinada como intervalo, em dias, entre a exérese cirúrgica e o óbito por qualquer causa (global) ou por causas associadas a progressão da neoplasia mamária (específica). Todos os animais tinham um acompanhamento de no mínimo um ano.

5.7 Análise estatística

Para a análise estatística foram utilizados os programas GraphPad Prism versão 8.2 e Jamovi 2.3.28. Para associar as variáveis qualitativas (imunofenótipos, tipos histológicos, grau de inflamação e intensidade de infiltrado inflamatório, presença de metástase para linfonodo regional, presença de necrose, tamanho tumoral e expressão de Cox-2) foram utilizados os testes Qui-quadrado ou Exato de Fischer, conforme indicado pelo software. O teste de Kolmogorov-Smirnov foi usado para testar a normalidade da amostra. Para associação de Ki-67 e contagem mitótica com tipos histológicos e grau de inflamação e infiltrado inflamatório foi feito o teste de Kruskal-Wallis, seguido pelo Teste Dunn ou Man Whitney. Em todos os casos, o $p < 0,05$ foi considerado significativo. A análise de sobrevida global e específica foi feita através de curvas de Kaplan Meier, e a comparação entre as curvas pelo teste de Long-rank. Na análise de sobrevida específica associada ao grau de inflamação, o grau II e III foram unificados, visto que poucas cadelas com neoplasias no grupo III tinham informação do seguimento. O modelo de regressão de Cox foi utilizado para análise univariada e multivariada,

levando-se em conta variáveis cuja significância em análise univariada foi $< 0,2$.

5.8 Aspectos éticos

Todos os procedimentos realizados neste trabalho estavam de acordo com os princípios éticos para uso de animais em experimentação aprovado pelo CEUA – UFMG (nº 11/2017, e foi aprovado em 29/03/2021).

6. RESULTADOS E DISCUSSÃO

Os resultados e a discussão serão apresentados em forma de artigos, que foram submetidos aos periódicos *Journal of Comparative Pathology* e *Veterinary Research Communications*.

ARTIGO I – PUBLICADO

Flecher MC, Balabram D, Salles YA, Souza FR, Estrela-Lima A, Nakagaki KYR, Cassali GD. Evaluation of immunophenotype and inflammation in canine mammary neoplasms with solid arrangement. J Comp Pathol. 2024;213:1-9. doi: 10.1016/j.jcpa.2024.06.004. - <https://pubmed.ncbi.nlm.nih.gov/39018987/>

Neoplastic disease

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Evaluation of immunophenotype and inflammation in canine mammary neoplasms with solid arrangement

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ARTICLE INFO

Keywords:

bitch

immunohistochemistry

Ki-67

mammary cancer

survival

tumour-infiltrating lymphocytes

ABSTRACT

Morphological and immunohistochemical studies of solid arrangement canine mammary carcinomas have shown that the different histological types may be characterized by proliferation of epithelial and/or myoepithelial cells. However, little is known about immunophenotypes and the importance of inflammation as prognostic factors in these neoplasms. The objective of the present study was to characterize the immunophenotype and degree of inflammation in the solid type of canine mammary neoplasm and to investigate their association with metastasis, Ki-67 index, tumour size, necrosis and survival. Sixty-five carcinomas with solid pattern, basaloid carcinoma, solid papillary carcinoma, malignant adenomyoepithelioma or malignant myoepithelioma were investigated. Luminal A, luminal B HER2 negative and HER2 positive, HER2 overexpressed and triple negative immunophenotypes were immunolabelled as were Ki-67 protein and cyclooxygenase-2 (Cox-2). Histological peritumoural and intratumoural inflammatory infiltrates were graded (distribution $\times$ intensity) and the presence of necrosis identified. We found a statistical difference between histological types and immunophenotypes, with MME and MAME having

a higher occurrence of luminal A, whereas most neoplasms had the negative HER2 luminal B immunophenotype. There was no correlation between immunophenotype and degree of peri- and intratumoural inflammation, nodal metastasis, necrosis or tumour size. An increased degree of peri- and intratumoural inflammation was significantly associated with lymph node metastasis, and more severe intratumoural inflammation was associated with the presence of tumour necrosis. Tumour size, Ki-67 index and Cox-2 score were not associated with inflammation in either peri- or intratumoural regions. No difference was observed in survival in relation to immunophenotypes or degree of inflammation, but the Cox regression model revealed that nodal metastasis influenced the risk of death.

1. Introduction

Solid carcinoma of the canine mammary gland is one of the most aggressive mammary neoplasms [1,2], often causing metastases to regional lymph nodes [3] and a high death rate after surgery [2,4,5]. Such neoplasms are histologically characterized by cell growth organized in nests, islands and cords without luminal structure and often with scarce stroma [3,4]. Several reclassifications have been made on the basis of the morphological characteristics [3,6] and immunophenotypic characteristics indicate these neoplasms may consist of epithelial and/or myoepithelial cells, which leads to differences in clinical evolution [7,8]. In this context, a recent study proposed dividing neoplasms previously called ‘solid carcinomas’ into solid arrangement neoplasms of the subtypes carcinoma with solid pattern (CSP), solid papillary carcinoma (SPC), basaloid carcinoma (BC), malignant adenomyoepithelioma (MAME), malignant myoepithelioma (MME) and neuroendocrine carcinoma [8].

In human medicine, the discovery of wide genetic variation in breast tumours and its influence on morphological, phenotypic and behavioural patterns prompted the creation of a classification according to molecular immunophenotypes [9]. Based on molecular

classification, the characterization of immunophenotypes for women was developed with the use of immunohistochemical markers, as it is a more widespread and inexpensive than the molecular technique. Following the 13th International Conference on Breast Cancer, a new definitions of intrinsic subtypes of breast cancer in women defined as luminal A, luminal B (HER2 positive and negative), HER2 overexpressed and triple negative were published [10]. Analogous studies have been carried out in bitches, as the association of tumour histological type with immunophenotype is important in better defining the prognosis [11,12]. However, despite following human guidelines, the immunophenotypic markers and classification used in veterinary medicine are not standardized, which makes evaluation of the results difficult [12–14].

Most malignant mammary neoplasms of bitches are characterized by immunolabelling of hormone receptors (oestrogen [ER] and progesterone [PR]) but absence of immunolabelling of human epidermal growth factor 2 (HER2), which classifies them as luminal [12–15]. According to the human classification [10] and the adapted classification for bitches [14], quantification of Ki-67 protein by immunohistochemistry (IHC) allows distinction between luminal A, when the neoplasm expresses <20% of protein marking, and luminal B, when the expression is $\geq 20\%$. These types of tumours are associated with better prognosis [11,12,14]. Mammary neoplasms in bitches may also express HER2 associated with positive oestrogen and progesterone receptors; such neoplasms called luminal B HER2 positive. They may also present with HER2 overexpressed or triple-negative immunophenotypes when none of these markers are expressed [14]. Neoplasms with a triple-negative immunophenotype present have a significantly larger size, higher Ki-67 index and shorter survival time [11–13].

Inflammation has been extensively studied and correlated with immunophenotype so that prognostic factors and therapeutic methods can be established for women with breast cancer. A higher number of tumour-infiltrating lymphocytes (TILs) in the tumour stroma among nests of

neoplastic cells is associated with higher histological grade, vascular invasion and metastasis to lymph nodes, higher expression of Ki-67 and lower hormone receptor expression in women [16]. However, women who have triple-negative neoplasms (TNs), overexpressed HER2 immunophenotypes and a higher number of TILs in the tumour stroma have better disease-free interval and survival when compared with those who have neoplasms of the luminal subtypes [17,18]. Higher numbers of TILs have been related to a better response to chemotherapy with longer disease-free time in TN patients [18]. In bitches, lymphocytes are the predominant cells in the mammary tumour microenvironment [19–21] and are present in higher numbers in neoplasms of higher histological grade and with lymphatic invasion [22]. However, few studies have correlated the degree of inflammation with the immunophenotype and specific histological types [11,22].

Cox-2 is an enzyme present in the tumour microenvironment that is associated with inflammatory cell infiltration [23]. In mammary cancer, high expression of Cox-2 is associated with increased angiogenesis, invasiveness, metastasis and a worse prognosis in women [24,25] and bitches [26–28].

Despite being included among the prognostic factors and assisting in the choice of treatment strategies for breast neoplasms in women, molecular immunophenotypes [29] and tumour inflammation [18,30,31] have undergone only limited investigation in animals [11,14,19,21]. The objective of the present study was to characterize the immunophenotypes and degree of inflammation in solid arrangement neoplasms of the canine mammary gland, and to associate those characteristics with prognostic factors such as Ki-67 index, Cox-2 expression, presence of regional lymph node metastasis, tumour size and presence of necrosis, as well as to evaluate the overall and specific survival of bitches.

2. Materials and methods

All procedures performed in this study were in accordance with the ethical principles for the use of animals in experimentation approved by Ethics Committees for the Use of Animals-Federal University of Minas Gerais (No. 11/2017).

2.1. Sample selection and clinical follow-up

From 2011 to 2022, 120 cases of solid arrangement mammary neoplasms of bitches were selected from the collection of the Comparative Pathology Laboratory of the Institute of Biological Sciences of Federal University of Minas Gerais, Pathology Laboratory of Vila Velha University, Celulavet Laboratory and Research Center on Mammary Oncology of Federal University of Bahia.

As inclusion criteria, samples came from bitches with available slides stained with haematoxylin and eosin (HE) and paraffin blocks and/or gelatinized slides for IHC. The neoplasms had been classified previously as ‘solid carcinoma’ or basaloid carcinoma by Cassali *et al* [32] and had a macroscopic description that included location and size. Cases without any information regarding the evaluation of regional lymph nodes (inguinal or axillary) and bitches with malignancy of non-mammary origin or another mammary histological type with a worse prognosis were excluded. Based on these criteria, 65 samples were included in the study and the histological slides were reassessed by two veterinary pathologists (GDC, MCF) and classified as solid arrangement neoplasms according to the criteria adopted by Cassali *et al* [32]. Subsequently, the neoplasms were reclassified into CSP, BC, SPC, MAME and MME according to morphological and phenotypic patterns, following the guidelines of Nakagaki *et al* [8]. Neuroendocrine carcinoma was not evaluated in the present study, as there were few cases and the samples were not suitable for the study. Regional lymph nodes were also evaluated for metastasis.

Tumours were graded according to the Nottingham System adapted for canine neoplasms [33], except for MAME and MME, as they do not fit the standardization of histological grading used for invasive carcinomas. Information on size of the neoplasms was provided by the laboratory from which the samples had come. Accordingly, tumour size was stratified into T1 (<3 cm), T2 (3–5 cm) and T3 (>5 cm), following the Tumour Node Metastasis classification of tumours [34]. As the information on distant metastasis assessment was incomplete, complete staging was not performed in this study. Necrosis was evaluated microscopically and classified as absent or present.

For the survival parameter, the time in days from the date of surgery until the last contact, or until the patient's death, with at least 1 year of follow-up, was calculated. Information was obtained from the responsible tutor and/or the veterinary clinician.

2.2. Immunohistochemistry for characterization of immunophenotypes

IHC was performed for ER and (PR), cell proliferation factor Ki-67 and HER2 in all 65 selected samples. Immunolabelling for Cox-2 was also performed on 63 samples. (Due to wear of the paraffin block during the study, two cases lacked sufficient material for this analysis). Four-µm thick sections were cut from the paraffin blocks and placed on gelatinized slides, followed by deparaffinization, dehydration in alcohol and xylol and manual immunolabelling for the markers according to validated protocols (Table 1). The secondary antibody and development system used was the commercial Novolink Polymer Detection System (Leica Biosystems, www.leicabiosystems.com). Evaluations were performed with the use of an Olympus BX-40 microscope coupled with a Spot Insight Color digital camera and with the aid of SPOT Capture Software-version 3.4.5(Spot Imaging, www.spotimaging.com). A scoring system was used for the evaluation of ER and PR labelling, with nuclear labelling being observed at any intensity (negative = <1%; + = 1–25% of labelled cells; ++ = 26–50% of

labelled cells; +++ = 51–75%; ++++ = >75% of labelled cells) [35]. Ki-67 was accounted for by nuclear labelling in 500 cells, in the Image J imaging system, from photomicrographs made in a hotspot under a $\times 40$ objective (2.37 mm^2), with a cutoff point of $\geq 20\%$ of cells labelled [14]. HER2 expression was evaluated following the guidelines for membrane marking score, with scores of 0, 1+ and 2+ considered negative, and 3+ positive [36]. Neoplasms were categorized into luminal A (ER and/or PR positive, HER2 negative and Ki-67 $< 20\%$), luminal B HER2 negative (ER and/or RE positive, HER2 negative and Ki-67 $\geq 20\%$), luminal B HER2 positive (ER and/or PR positive, HER2 positive and any Ki-67), HER2 overexpressed (ER and RP negative and HER2 positive), and triple negative (TN) (ER, PR, and HER2 negative) (Table 2). Normal mammary gland was used as positive control tissue while a buffer solution replaced the primary antibodies as a negative control. Cox-2 assessment was based on semiquantitative evaluation of cytoplasmic immunolabelling of tumour cells using distribution (0 = absent, 1 = $< 10\%$ labelled cells, 2 = 10 to 30% labelled cells, 3 = 31 to 60% labelled cells, 4 = $> 61\%$ labelled cells) and intensity (0 = absent, 1 = weak (+) label, 2 = moderate (++) label, and 3 = strong (+++) scores. To establish the overall score, the distribution score was multiplied by the intensity score [26].

2.3. Evaluation of inflammatory infiltrate

The grading of peritumoural and intratumoural inflammatory infiltrates was done on an HE-stained slide. In the case of a neoplasm $> 2 \text{ cm}$ in diameter, the slide with a fragment approximately 2 cm in area with well- preserved cellular characteristics, and less necrosis, haemorrhage or artefact was selected. The evaluation was performed under $\times 10$ and $\times 20$ lenses with an Olympus BX-40 microscope. Peritumoural inflammation was defined as the presence of lymphocytes in the connective tissue of the capsule or, in the absence of a capsule, the tumour margin without direct contact with the neoplasm. Intratumoural inflammation was defined as

the presence of lymphocytes in the fibrous connective tissue between and within nests of neoplastic cells, except those in areas of necrosis. Classification of peri- and intratumoural inflammation, ranging from I to III, was made through a distribution and intensity score following established methodology in veterinary medicine [19,22]. (Table 3).

2.4. Statistical analysis

GraphPad Prism version 8.2 and Jamovi 2.3.28 were used for statistical analysis. Chi-square or Fischer's exact tests were used, as indicated by the software, to associate immunophenotypes, histological types, degree of inflammation, regional lymph node metastasis and presence of necrosis. As for the association of Ki-67 with histological type and degree of inflammation, the Kruskal–Wallis test was performed, followed by a Dunn test. In all cases, $P < 0.05$ was considered significant. Analysis of overall and specific survival was made using Kaplan–Meier curves, and comparison between the curves was obtained by the Long-rank test. In this specific survival analysis associated with intratumoural inflammation, degrees II and III were unified, since few bitches with neoplasms in group III presented with follow-up information. The Cox regression model was used for univariate and multivariate analysis, taking into account variables whose significance in univariate analysis was < 0.2 .

3. Results

One-hundred and twenty cases with a diagnosis of solid carcinoma of the canine mammary gland were evaluated. However, after selection based on the inclusion and exclusion criteria, 65 samples of solid arrangement neoplasms were evaluated. Neoplasms were classified as MME (8/65, 12.3%), SPC (8/65, 12.3%), CSP (10/65, 15.4%), BC (19/65, 29.2%) or MAME (20/65, 30.8%). Bitches with these neoplasms were 3 to 19 years old, with a mean of 11 years old (standard deviation ± 3.3).

In the classification by immunophenotype, 12/65 (18.5%) neoplasms were luminal A, 47/65 (72.3%) were luminal B HER2 negative, 6/65 (9.2%) were luminal B HER2 positive and no neoplasms were either HER2 overexpressed or TN. The association of the variables (histological types, metastasis to regional lymph nodes, size, necrosis) with the immunophenotypes of solid arrangement neoplasms are shown in Table 4. When comparing the immunophenotype between histological types, MME and MAME were represented by more of luminal A neoplasms (41.7% each) than other types of solid neoplasm. CSPs, on the other hand, were represented by a higher number of negative luminal B HER2 subtype and no luminal A. Luminal B HER2-negative immunophenotype was also more abundant in histological types BC, MAME and SPC neoplasms (Fig. 1A, $P = 0.01$). Ki-67 immunolabelling was $\geq 20\%$ in 53 (81.5%) of the 65 neoplasms evaluated. The median expression values of Ki-67 were 33% for CSP (21–60%), 30% for BC (8–50%), 28% for SPC (2–89%) and 28% for MAME (8–51%). Immunoexpression was lowest in MMEs (19%; 12–24%). A statistical difference between histological types ($P = 0.007$) was found. In the analysis of multiple comparisons, statistical difference was found between Ki-67 expression in MME and CSP ($P = 0.009$) and between MME and BC ($P = 0.008$, Fig. 1B).

In the cases evaluated, 23/65 (35.4%) that had regional lymph node metastasis were identified. Of these, 20 (87%) were neoplasms with luminal B immunophenotype (HER2 negative and HER2 positive), but no statistical difference in regional metastasis was found between immunophenotypes ($P = 0.58$), and there was no association of immunophenotype with either tumour size ($P = 0.07$) or for necrosis ($P = 0.92$).

Inflammation in neoplasms was characterized by a predominance of mononuclear cells, mainly lymphocytes, followed by plasma cells and macrophages. Inflammation in the peritumoural area consisted only of degrees I and II (Fig. 2A and B), while in the intratumoural area 4/65 (6.2%) neoplasms had inflammation of degree III, in addition to degrees I and II (Fig.

2C–E). The variables associated with degree of inflammation are shown in Table 5. Of the four neoplasms that had degree III inflammation, two were CSP. However, no statistical significance of peritumoural ($P = 0.49$) or intratumoural ($P = 0.28$) inflammation with histological types was found. When inflammation was associated with the immunophenotype, luminal A neoplasms had only degree I and II inflammation in the peri- and intratumoural areas whereas 3/47 (6.4%) of HER2-negative and 1/6 (16.7%) of HER2-positive luminal B neoplasms had degree III inflammation in the intratumoural area. Even though luminal B neoplasms exhibited grade III intratumoural inflammation, unlike luminal A neoplasms, there was no association of peritumoural ($P = 0.17$) or intratumoural ($P = 0.43$) inflammation with immunophenotype.

Neoplasms with degree I peritumoural inflammation had a lower occurrence of metastases to regional lymph nodes (10/40, 25%) than those with degree II inflammation (13/25, 52%, $P = 0.03$). When lymph node metastasis was associated with intratumoural inflammation, 9/39 (23.01%) of degree I neoplasms, 10/22 (45.5%) of degree II and 4/4 (100%) of degree III had lymph node metastasis ($P = 0.004$, Fig. 3).

All neoplasms with degree III intratumoural inflammation and 81.8% of those with degree II had necrosis ($P < 0.001$). Intratumoural inflammation was not associated with the Ki-67 index ($P = 0.36$) or tumour size ($P = 0.13$). In addition, no relationship was found between Ki-67 index ($P = 0.23$), necrosis ($P = 0.36$) or size ($P = 0.14$) and peritumoural inflammation.

Immunolabelling of Cox-2 was performed on 63 neoplasms and was positive in neoplastic cells in 19/63 (30.6%). Of these, 17 had a Cox-2 score < 6 , with no association with peri- ($P = 0.58$) or intratumoural ($P = 0.23$) inflammation.

Of the 52 bitches with follow-up information, 21 (40.4%) died from mammary cancer, 15 (28.8%) died from other causes and 16 (30.8%) were alive at the end of the study period. The median overall survival was 518 days (95% CI, 406–630 days), while specific survival was 692 days (95% CI, 565–819 days). No statistical difference was found between the

immunophenotypes of neoplasms and overall ($P = 0.28$) and specific ($P = 0.63$) survival of bitches (Fig. 4 A and B). However, bitches with HER2-positive luminal B neoplasms had a lower median overall survival (181 days) and specific survival (80 days) when compared with luminal A and HER2-negative luminal B tumours, which were associated with more than 400 days in overall survival and 600 days in specific survival. Peritumoural ($P = 0.23$) or intratumoural ($P = 0.7$) inflammation was not associated with overall survival of bitches. For the Kaplan–Meier curve, degrees II and III of intratumoural inflammation were unified, as few animals with degree III had their cause of death determined. Bitches with degree I peritumoural inflammation had a specific survival of 670 days, while the value for those with degree II was 395 days ($P = 0.22$). In the intratumoural evaluation, the median survival time of bitches with degree I was 670 days and among those with degrees II/III it was 575 days ($P = 0.7$) (Fig. 4 C and D).

In the univariate analysis by the Cox regression method, the Ki-67 index ($P = 0.02$, hazard ratio [HR] = 1.03, confidence interval [CI] 95% CI 1.00–1.05), lymph node metastasis ($P = 0.001$, HR = 7.4) and degree III intratumoural inflammatory infiltrate in relation to degree I ($P = 0.008$, HR = 6.1, CI = 1.5–23.0) were associated with the risk of death from neoplasia. In the multivariate analysis of Cox regression, only metastasis to regional lymph nodes continued to present a higher risk ratio in relation to death ($P = 0.02$, HR = 5.6, CI = 2.88–19.2), and was considered an independent factor for death (Table 6).

4. Discussion

In the present study, solid arrangement neoplasms with HER2-negative luminal B molecular immunophenotype were predominant. All neoplasms were positive for hormone receptors, falling within the luminal immunophenotype. Most neoplasms had a high Ki-67 index ($\geq 20\%$) and did not express HER2. Even with the divergence of methodologies used for

the categorization of immunophenotypes in mammary neoplasms of bitches [11,12,14,15], a predominance of mammary neoplasms of immunophenotype luminal A has been reported [12,13,37] and only one study described a higher frequency of TN neoplasms in bitches [11]. The results of a study of various histological types of canine mammary neoplasms were consistent with the present study, and of the 30 carcinomas classified as solid, 27 (90%) were luminal subtype but were not subdivided into A and B [14].

In animals, the cutoff point used to classify Ki-67 in mammary neoplasms is currently $\geq 20\%$ [14,32], which also follows the division of luminal A and B subtypes described by Goldhirsch *et al* [10]. Some protocols for the classification of immunophenotypes in animals do not include analysis of Ki-67 expression [12,37], but in the classification of immunophenotypes in women, patients with a luminal A neoplasm had a better prognosis and longer survival time than women who had luminal B neoplasms, indicating the importance of Ki-67 for distinguishing between these two cancer subtypes [38,39]. MME had the lowest median score for Ki-67, which is an important factor in classifying most of them as luminal A.

Only six cases had immunolabelling for HER2 and as they were ER and/or PR positive, no case of overexpressed HER2 immunophenotype was identified. Mammary neoplasms with overexpressed HER2 immunophenotype are rare in bitches [11,15] and the correlation of their expression with morphological factors such as histological grade, mitotic count, nuclear pleomorphism and Ki-67 index is variable [40]. Furthermore, the amplification of *ERBB2*, the gene encoding the HER2 protein, is less common in bitches than in women [41]. Therefore, the discussion is centered around whether this oncoprotein can be considered a prognostic factor and whether it influences the progression of malignant mammary neoplasms in bitches [42,43].

In human patients, the correlation between immunophenotype and lymph node status is highly variable depending on the histological type, but some studies have associated a lower incidence of metastases in luminal A than in luminal B or overexpressed HER2 neoplasms

[44,45]. In bitches, a lower occurrence of metastasis and vascular invasion in luminal neoplasms compared with TNs has been reported [41]. No association of immunophenotype with lymph node metastasis was found in the present study, but the presence of only luminal neoplasms and no overexpressed HER2 or TN may have influenced this result. However, other studies of bitches with various histological and immunophenotypes of mammary neoplasms also found no statistical association between immunophenotype and lymph node metastasis [37]. In canine studies, tumour size and necrosis were not statistically associated with immunophenotype [14,37], corroborating the findings of our study.

Inflammation is known to have pro- or anti-tumour roles depending on the immune cells present and the cytokines and chemokines secreted by them, the phenotype and location of inflammation, and the stage of the neoplasm [46,47]. We analysed the degree and site of inflammatory infiltration in the HE-stained slides, as this can be readily done in routine veterinary pathology and can be compared with the evaluation of TIL in mammary neoplasms of women [31,48,49]. In the present study, we evaluated inflammation without quantifying each cell type separately, but found a predominance of lymphocytes in the inflammatory infiltrate.

No statistical difference between peri- or intratumoural inflammation and histological type or immunophenotype was found. A similar study in bitches also did not find a statistical association between the degree of inflammation and histological type or immunophenotype. However, the degree of inflammation was higher in neoplasms with prognostically worse immunophenotypes, such as overexpressed HER2 and TN [22]. In his study, we observed that some histological types (CSP, BC and MAME) and luminal B neoplasms (HER2 negative and HER2 positive) had degree III inflammation in the intratumoural area, but not in the peritumoural area. Luminal A neoplasms in women are associated with lower expression of genes related to immune mechanisms and proportionally had a lower number of TILs than luminal B neoplasms [50]. Both in women and bitches, immunophenotypes with a worse

prognosis, such as TN, seem to have greater proliferative activity and higher mutagenic activity and, therefore, induce the strongest immune response [42,17]. The predominance of degrees I and II inflammation in the present study can be explained by the fact that the neoplasms were exclusively of the luminal type.

The laboratories from which the samples came carry out a large number of histopathological examinations of mammary neoplasms in Brazil, but the lack of information on some of the bitches limited the availability of samples for inclusion in the study. This may have influenced some of the statistical analyses.

The presence of lymph node metastasis was related to higher degrees of peri- and intratumoural inflammation), in agreement with reports for women [16,51]. In bitches, increased lymphocyte infiltration in neoplasms was significantly associated with lymphatic invasion [22].

The presence of necrosis was proportionally associated with intratumoural inflammation. During the process of necrosis, neoplastic cells recruit immune cells and these may assist in the phagocytosis of cell fragments and also secrete inflammatory cytokines and growth factors for angiogenesis and proliferative stimulation [52]. No statistically significant association was found between inflammation in peri- or intratumoural locations and Ki-67 rate, which is different from what has been reported in women [16]. We found that higher Ki-67 expression increased the risk of death in bitches with solid arrangement neoplasms.

In the present study, Cox-2 expression in neoplastic cells was small and most neoplasms had low marker scores that were unrelated to the degree of inflammation. Higher expression of Cox-2 has been associated with more severe inflammation in mammary neoplasms in bitches [28]. However, different methodologies [26,27,28] were used to evaluate Cox-2 expression by IHC, which may generate divergent results.

No association was found between the immunophenotype of neoplasms and degree of inflammation, nor between immunophenotype and inflammation and survival according to the Kaplan Meier curve. In women with TN neoplasms, higher numbers of TILs in tumour stroma is associated with better specific survival [53] and reduced cancer recurrence [17,31]. In the univariate analysis with the Cox regression method, we found that bitches with neoplasms with degree III inflammation had a risk of death six times higher than those that had neoplasms with degree I inflammation, but this variable is not an independent factor. In women, the response to therapy is better in those with neoplasms with higher numbers of TILs [17,31]. However, there are no analogous studies in bitches.

Another important result of the present study is that a higher degree of peri- and intratumoural inflammation is associated with metastasis, which is an independent factor for the risk of death of bitches with solid arrangement neoplasms, and which agrees with a study on solid neoplasms of the canine mammary gland [8]. Lymph node metastasis of other histological types of mammary neoplasms in bitches has been also associated with shorter survival time and higher risk of death [19,54].

5. Conclusion

The most frequent immunophenotype in solid arrangement neoplasms was luminal B HER2 negative, with CSP being the most frequent type with this molecular profile. MMEs had a greater predominance of the luminal A immunophenotype and the lowest median expression of Ki-67.

The degree of inflammation was not associated with histological type and immunophenotype. However, animals with greater peri- and intratumoural inflammation had a significantly higher rate of metastasis to regional lymph nodes, which was an independent factor for the risk of death. A relationship was observed between histological types and

immunophenotypes, but not between these parameters and degree of inflammation. However, inflammation was directly associated with metastasis, which in turn increased the risk of death.

Funding

This study was funded by the National Council for Scientific and Technological Development (CNPq), the Minas Gerais Research Foundation – FAPEMIG, the Minas Gerais Network for Translational Research in Immunobiologicals and Biopharmaceuticals in Cancer (REMITRIBIC, RED-00031-21) and the Coordination for Personal Improvement of Higher Education (CAPES), Brazil.

Acknowledgments

We thank the tutors and veterinarians for providing the necessary information to carry out this study.

Statement of author contributions

Mayra C. Flecher: Conceptualization; Methodology; Investigation; Writing – review & editing, original draft; Visualization; Project administration. Yasmin A. Salles: Investigation. Fernanda R. Souza: Investigation. Alessandra E. da Silva Lima: - Investigation. Débora Balabram: Formal analysis; Writing – review & editing. Karen Y.R. Nakagaki: Conceptualization; Methodology; Writing – review & editing; Project administration. Geovanni D. Cassali: Conceptualization; Methodology; Investigation; Writing – review & editing, original draft; Funding acquisition; Project administration.

Declaration of competing interests

The authors declared no conflicts of interest in relation to the research, authorship or publication of this article.

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Table 1

Details of the antibodies and antigen retrieval used in the study

Antibody	Clone	Dilution	Manufacturer	Incubation	Antigen retrieval
ER	Ep1	Ready for use	Dako (www.agilent.com)	Overnight	
PR	hPRa2	1:50	Thermo Fisher (www.thermofisher.com)	Overnight	
Her-2	c-erb- 2	1:200	Thermo Fisher	Overnight	Wet heat, citrate pH 6.0
Ki-67	mib 1	1:50	Dako	Overnight	
Cox-2	Sp21	Ready for use	Invitrogen (www.thermofisher.com)	Overnight	

Table 2

Classification of immunophenotypes of neoplasms of the canine mammary gland neoplasms used in

Immunophenotypes	Immunolabelling	this study
Luminal A	ER and/or PR positive, HER2 negative and Ki-67 <20%	
Luminal B HER2 negative	ER and/or RE positive, HER2 negative and Ki-67 $\geq$ 20%	
Luminal B HER2 positive	ER and/or PR positive, HER2 positive and any Ki-67	
HER2 overexpressed	ER and RP negative and HER2 positive	
Triple negative	ER, PR, and HER2 negative	

ER: oestrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor 2

Table 3

Guidelines for determining the degree of peri- and intratumoural inflammation in solid arrangement neoplasms

Distribution		Score
Focal	1–3 foci of inflammatory infiltrate	1
Multifocal	>3 foci of inflammatory infiltrate	2
Diffuse	Tumour-wide distribution	3
Intensity		
Discrete		1
Moderate		2
Intense		3

Grade = distribution × intensity.

Degree I, score 1 to 3; degree II, score 4 to 6; degree III, score 7 to 9.

Table 4

Morphological characteristics of solid arrangement neoplasms of the canine mammary gland, according to immunophenotype

Variables	Luminal A (n = 12) (n/%)	Luminal B HER-2 negative (n = 47) (n/%)	Luminal B HER-2 positive (n = 6) (n/%)	P <0.05*
Histological type				
CSP	0 (0%)	9 (19.1%)	1 (16.7%)	0.01*
SPC	1 (8.3%)	5 (10.7%)	2 (33.3%)	
BC	1 (8.3%)	17 (36.2%)	1 (16.7%)	
MME	5 (41.7%)	3 (6.3%)	0 (0%)	
MAME	5 (41.7%)	13 (27.7%)	2 (33.3%)	
Nodal metastasis				
Present	3 (20%)	17 (36.2%)	3 (50%)	0.58
Absent	9 (75%)	30 (63.8%)	3 (50%)	
Tumour size				
T1 (<3 cm)	2 (16.7%)	21 (44.7%)	2 (33.3%)	0.07
T2 (3–5 cm)	2 (16.7%)	12 (25.5%)	1 (16.7%)	
T3 (>5 cm)	8 (66.6%)	14 (29.8%)	3 (50%)	
Necrosis				
Present	7 (58.3%)	26 (55.3%)	4 (66.7%)	0.92
Absent	5 (41.7%)	21 (44.75%)	2 (33.3%)	

CSP, carcinoma with solid pattern; SPC, solid papillary carcinoma; BC, basaloid carcinoma; MME, malignant myoepithelioma; MAME, malignant adenomyoepithelioma.

* Statistically significant when $P < 0.05$.

Table 5

Association of morphological characteristics and degree of inflammation in solid arrangement neoplasms of the canine mammary gland

	Peritumoural inflammation				Intratumoural inflammation			
Variables	Grau I (n = 40)	Grau II (n = 25)	Grau III (n = 0)	P (<0.05)*	Grau I (n = 39)	Grau II (n = 22)	Grau III (n = 4)	P (<0.05)*
Histological type								
CSP	4 (10%)	6 (24%)	0 (0%)	0.49	3 (7.7%)	5 (22.7%)	2 (50%)	0.28
SPC	6 (15%)	2 (8%)	0 (0%)		6 (15.4%)	2 (9.1%)	0 (0%)	
BC	15 (37.5%)	4 (16%)	0 (0%)		14 (35.9%)	4 (18.2%)	1 (25%)	
MME	6 (15%)	2 (8%)	0 (0%)		6 (15.4%)	2 (9.1%)	0 (0%)	
MAME	9 (22.5%)	11(44%)	0 (0%)		10 (25.6%)	9 (40.9%)	1 (25%)	
Nodal metastasis								
Present	10 (10%)	13 (52%)	0 (0%)	0.03*	9 (23.1%)	10 (45.5%)	4 (100%)	0.004*
Absent	30 (90%)	12 (48%)	0 (0%)		30 (76.9%)	12 (54.5%)	0 (0%)	
Tumour size								
T1 (<3 cm)	18 (45%)	7 (28%)	0 (0%)	0.14	19 (48.7%)	5 (22.7%)	1 (25%)	0.13
T2 (3–5 cm)	9 (22.5%)	6 (24%)	0 (0%)		8 (20.5%)	6 (27.3%)	1 (25%)	
T3 (>5 cm)	13 (32.5%)	12 (48%)	0 (0%)		12 (30.8%)	11 (50%)	2 (50%)	
Necrosis								
Present	21 (52.5%)	16 (64%)	0 (0%)	0.36	15 (38.5%)	18 (81.8%)	4 (100%)	0.001*
Absent	19 (47.5%)	9 (36%)	0 (0%)		24 (61.5%)	4 (18.2%)	0 (0%)	
Cox-2								
Positive	11 (28.2%)	8 (34,8%)	0 (0%)	0.58	9 (23.7%)	9 (45%)	1 (25%)	0.23
Negative	28 (71.8%)	15 (65,2%)	0 (0%)		29 (76.3%)	11 (55%)	3 (75%)	
Immunophenotype								
LA	9 (25%)	3 (75%)	0 (0%)	0.17	6 (50%)	6 (50%)	0 (0%)	0.43
LB HER-2–	32 (68.1%)	15 (31.9%)	0 (0%)		29 (61.7%)	15 (31.9%)	3 (6,4%)	
LB HER-2+	3 (50%)	3 (50%)	0 (0%)		4 (66.6%)	1 (16.7%)	1 (16,7%)	

CSP, carcinoma with solid pattern; SPC, solid papillary carcinoma; BC, basaloid carcinoma; MME, malignant myoepithelioma; MAME, malignant adenomyoepithelioma, LA, luminal A; LB HER-2–, luminal B negative; LB HER-2+, luminal B HER-2 positive.

* Statistically significant when $P < 0.05$.

Table 6

Univariate and multivariate Cox proportional-hazards regression analyses comparing Ki-67 index, nodal metastasis, and intratumoral inflammation in solid arrangement neoplasms of the canine mammary gland

<i>Variables</i>	<i>HR</i>	<i>95% CI</i>	<i>P value</i>
Univariate analyses			
Ki-67 index (%)	1.03	1.00–1.05	0.02*
Nodal metastasis	7.4	2.8–19.23	0.001*
Intratumoural inflammation			
II versus I	2	0.78–5.04	0.14
III versus I	6.1	1.59–23.0	0.008*
Multivariate analysis			
Ki-67 index (%)	1	0.98–1.04	0.4
Nodal metastasis	5.6	1.8–17.5	0.002*
Intratumoural inflammation			
II versus I	1	0.39–2.92	0.89
III versus I	2.3	0.53–10	0.26

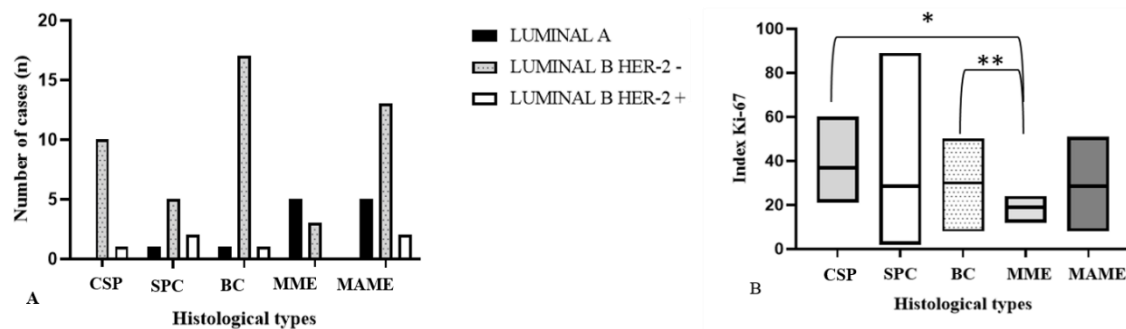
Figure legends

Fig 1. Distribution of Ki-67 immunophenotypes and immunoexpression, solid arrangement neoplasms, canine mammary gland. **(A)** Comparison of number of immunophenotypes between histological types of solid arrangement canine mammary neoplasms. Fischer test ($P = 0.01$). **(B)** Comparison of Ki-67 index between histological types of solid canine mammary gland neoplasms; Kruskal–Wallis and Dunn's post test (* $P = 0.009$; ** $P = 0.008$). CSP, carcinoma

with solid pattern; SPC, solid papillary carcinoma; BC, basaloid carcinoma; MME, Malignant Myoepithelioma; MAME: Malignant Adenomyoepithelioma).

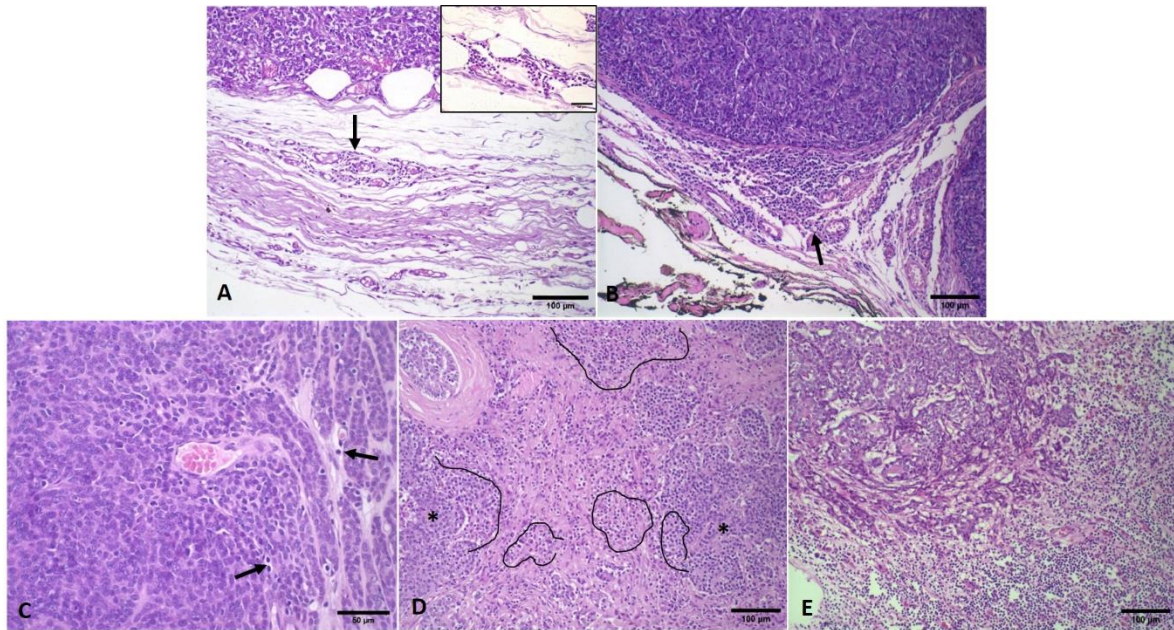


Fig 2. Solid arrangement neoplasms, mammary gland, dogs. **(A)** Focal area of mild inflammatory peritumoural infiltrate (arrow); degree I inflammation. Inset: Detail of peritumoural inflammatory infiltrate. HE. **(B)** Focus of peritumoural inflammation of moderate cellularity (arrow); degree II inflammation. HE. **(C)** Sparse intratumoural inflammatory cells (arrows), admixed with neoplastic cells, characteristic of degree I inflammation. HE. **(D)** Foci of inflammatory cell infiltration (contoured) in stroma and in contact with nests of neoplastic cells (*); degree II inflammation. HE. **(E)** Intense and diffuse inflammation in direct contact with nests of neoplastic cells; degree III inflammation. HE.

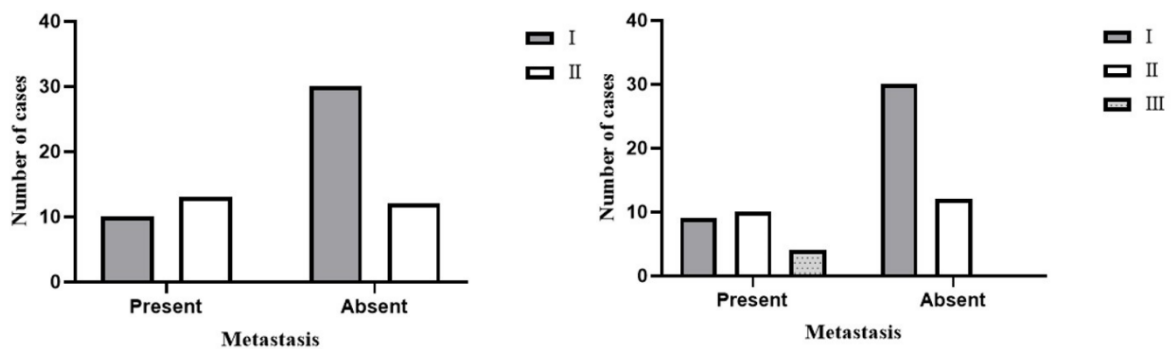


Fig 3. Comparison of regional lymph node status with degree of peri- and intratumoural inflammation in solid arrangement mammary neoplasms. Fischer test. **(A)** Neoplasms with degree I peritumoural inflammation had lower occurrence of metastases than those with degree II ($P = 0.03$). **(B)** Metastasis was associated with increased inflammation (grades II and III) in neoplasms ($P = 0.004$).

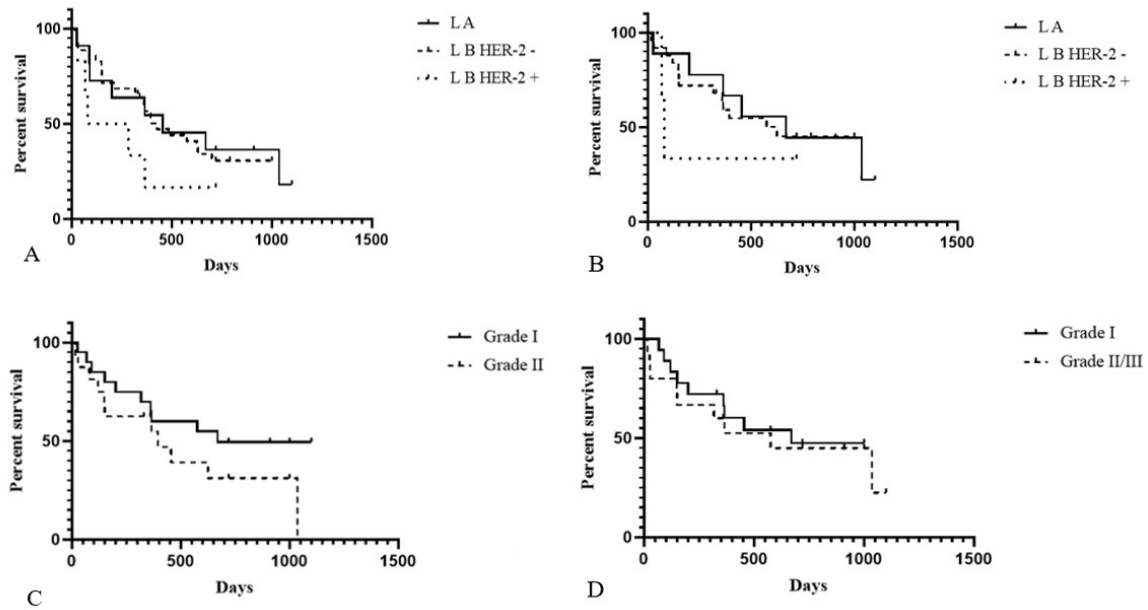


Fig 4. Kaplan-Meier survival curves of bitches with solid arrangement neoplasms of the mammary gland. P values refer to log-rank teste. **(A)** Overall survival associated with immunophenotypes ($P = 0.28$). **(B)** Specific survival associated with immunophenotypes ($P = 0.63$). LA, luminal A; LB, luminal B. **(C)** Specific survival associated with degree of peritumoural inflammation ($P = 0.22$). **(D)** Specific survival associated with the degree of intratumoural inflammation ($P = 0.7$).

Tumor-infiltrating inflammatory cells in canine mammary neoplasms with solid arrangement: a prognostic factor?

Tumor-infiltrating lymphocytes and neutrophils in canine mammary neoplasms with solid arrangement: a prognostic factor?

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Abstract

In canine mammary neoplasms, greater inflammation associates with higher histological grade, lymphatic invasion and metastases. This retrospective study assessed the intensity of peri and intratumoral tumor-infiltrating lymphocytes (TILs) and tumor-associated neutrophils (TANs) on histological slides, CD3⁺ and CD79⁺ lymphocytes in immunohistochemistry in canine mammary neoplasms with solid arrangement, and associate it with histological types, immunophenotype, prognostic factors, cyclooxygenase-2 (Cox-2) and overall and specific survival. Sixty-one neoplasms with solid arrangement were analyzed, and reclassified as malignant myoepitheliomas 6 (9.8%), solid papillary carcinomas 8 (13.1%), carcinomas with solid pattern 9 (14.8%), basaloid carcinomas (BC) 19 (31.1%), and malignant adenomyoepitheliomas 19 (31.1%). Immunohistochemistry was performed to establish the immunophenotypes and quantify TCD3⁺, BCD79⁺ lymphocytes, Ki-67 and Cox-2. The count of intra and peritumoral TILs and TANs were performed from histological slides, and TCD3⁺, BCD79⁺ lymphocytes in immunohistochemistry, and by the median, were dichotomized with low and high cell infiltration. BCs were the histological type with the lowest intensity of intratumoral TILs ($p=0,02$). Luminal B neoplasms showed significant higher intensity of intratumoral TCD3⁺ than luminal A. The amount of peritumoral TCD3⁺ and BCD79⁺ were proportional and statistically correlated to Ki-67 and mitosis. High infiltration of intratumoral BCD79⁺ lymphocytes is related to nodal metastasis ($p=0,03$), with metastasis being an independent prognostic factor for cancer-related death. Intratumoral TILs and TCD3⁺ are associated with shorter survival time. Therefore, lymphocyte infiltration, especially intratumoral, is likely to be an important feature in the progression of cancer and influence the survival in bitches with solid arrangement neoplasms.

Keywords: intratumoral, prognostic factor, CD3, CD79, neutrophils, metastasis.

Introduction

The characterization of tumor microenvironment through the evaluation of infiltrating leukocytes is already established for some types of neoplasms in the human species, such as colorectal carcinomas (Klintrup et al. 2005; Mlecnik et al. 2011; Galdiero et al. 2019), oral and cutaneous squamous cell tumors (Shimizu et al. 2018; Stravodimou et al. 2018), renal, head and neck, hepatocellular, pancreatic tumors (Shen et al., 2014), mammary (Zhu et al. 2015; Yu et al. 2016) and urothelial bladder cell tumors (Liu et al. 2018).

The presence of tumor-infiltrating lymphocytes (TILs) and their subtypes is associated with morphological (histological grade, size, pleomorphism and mitotic count) and immunophenotypic characteristics of breast tumors. Thus, TILs are considered prognostic and predictive factors in women's breast cancers (Denkert et al. 2010; Mahmoud et al. 2011; Mohammed et al. 2013; Gao et al. 2020;). In women, TILs tend to be present in a greater proportion in neoplasms without expression of hormone receptors and which show HER-2 overexpression and a higher percentage of Ki-67 (Lee et al. 2015; Wang et al. 2016; Koletsa et al. 2020). Also, greater inflammation in triple negative neoplasms (TN) and with HER-2 overexpression, especially in the intratumoral area, is associated with better response to chemotherapy treatment (Denkert et al. 2010; Lee et al. 2015; Gao et al. 2020). In bitches, the highest intensity of TILs is observed in neoplasms of higher histologic grade, with lymphatic invasion (Kim et al. 2013; Muscatello et al. 2022) and shorter survival time, a characteristic that is considered an independent prognostic factor. In addition, the highest percentage of B cells (CD79⁺) was seen in carcinoma in mixed tumor with metastasis to lymph nodes, when compared to carcinoma in mixed tumor without metastasis to lymph nodes (Estrela-Lima et al. 2010). However, little is known about the influence of inflammation and the relationship of inflammation and immunophenotypes in mammary neoplasms in bitches. Kim et al. (2013) found no relationship between TILs and molecular subtypes and Abadie et al. (2018) observed

that peritumoral inflammation is an independent prognostic factor for bitches with triple-negative tumors.

Few studies approach tumor-associated neutrophils (TANs) in women breast cancer and most of those studies are done *in vitro* and in animal models. As with lymphocytes, depending on the type of cytokine or receptors present in the microenvironment, neutrophils may be associated with a longer or shorter survival time (Queen et al. 2005; Timaxian et al. 2021). Neoplasms with positive for estrogen receptors, in advanced stages, show an increase in the amount of TGF- β in the microenvironment, favoring the chemotaxis and polarization of N2 neutrophils, which favor neoplastic dissemination and pre-metastatic niche formation (Vasquez Rodriguez et al. 2017). In the few studies done on women, the increase in the average amount of neutrophils is associated with TN and HER-2 positive tumors (Soto-Perez-de-Celis et al. 2017) and high density of TANs is associated with shorter disease-free time (Kwantwia et al. 2021). In bitches, the amount of neutrophils was higher in carcinoma in mixed tumors and tubular carcinomas compared to solid carcinomas and benign mixed tumors (Souza et al. 2018).

Although the quantification of TILs in H&E slides is an important parameter and is associated with prognostic and predictive factors in breast cancer in women, it does not allow for the identification of lymphocyte subtypes, which are fundamental for understanding the immune response to cancer. In the present study, we quantified tumor infiltrating lymphocytes (TILs) and tumor-associated neutrophils (TANs) in the peri and intratumoral areas, on histological slides de H&E, and on slides with immunohistochemistry for CD3⁺ and CD79⁺ lymphocytes. Afterwards, we dichotomized high and low intensity inflammations to associate with the histological types of neoplasms with solid arrangement, immunophenotypes, prognostic factors (tumor size, regional lymph node metastasis, Ki-67 index and mitotic count), Cox-2 immunostaining and overall and specific survival time of bitches.

Material and Methods

Sample selection and classification and clinical follow-up

The criteria for sample selection, classification of neoplasms, and clinical follow-up of the female dogs were based on previous work (Flecher et al. 2024). Sixty-one canine mammary neoplasms with solid arrangement, diagnosed between 2011 and 2022, were selected from the collection of the Comparative Pathology Laboratory of the Institute of Biological Sciences of Federal University of Minas Gerais (UFMG), the Pathology Laboratory of Vila Velha University (UVV), Celulavet Laboratory and Center for Research in Mammary Oncology of Federal University of Bahia (UFBA). Only samples from bitches previously diagnosed with neoplasms classified as solid carcinoma (Zapulli et al. 2019) or basaloid carcinoma (Cassali et al. 2020), which presented macroscopic description information with location and size of neoplasms and evaluation of regional lymph nodes, were included. Female dogs that presented with other more aggressive mammary-origin neoplasms associated with shorter survival times (Rasotto et al. 2017; Cassali et al. 2020), those that presented with non-mammary malignant neoplasms, and those that had been treated prior to surgery were excluded from the study.

From these criteria, histological slides stained by Hematoxylin and Eosin (H&E) were re-evaluated by two veterinary pathologists (GDC, MCF) and reclassified into carcinomas with solid pattern (CSP), basaloid carcinomas (BC), solid papillary carcinomas (SPC), malignant adenomyoepitheliomas (MAME) and malignant myoepitheliomas (MME) by morphological and phenotypic patterns (Nakagaki et al. 2022). Neuroendocrine carcinomas were not evaluated in the present study due to the small sample number. In all 61 cases the regional lymph nodes were also evaluated for the detection of metastasis. As MAMEs and MMEs do not fit the standardization of histological grading used for invasive carcinomas, the grading system was not used in this study. Information on neoplasm size was provided by the laboratory where the samples came from, and tumor size was stratified into T1 (< 3 cm), T2 (between 3 and 5 cm) and T3 (> 5 cm), following the TNM system (Owen 1980). For neoplasms larger than T2, more

than one histological slide was evaluated; the slide containing the best sample of approximately 2.0 cm with less necrosis and fixation and crushing artifacts was selected. Mitotic counting was performed in 10 fields (2.37mm^2) at the tumor periphery. As information on distant metastasis assessment was incomplete, the complete staging was not performed in this study.

The time in days from the date of surgery until the death of the dog from any cause and from neoplasia-related cause was calculated for overall survival and specific survival, respectively. The follow-up was carried out for a period of at least one year. Information was obtained from the responsible tutor and/or the veterinary clinician.

Immunohistochemistry

Four micrometers thick sections were obtained from the paraffin blocks and placed on gelatinized slides, followed by deparaffinization, dehydration in alcohol and xylol, and manual staining for the markers following previously validated protocols. The antigenic recovery was done in moist heat, in citrate pH 6.0. Novolink[®] kit solution (Leica) was used as the secondary antibody and detection system. Evaluations were performed under an Olympus BX-40 microscope coupled with a Spot Insight Color digital camera and with the aid of SPOT capture software version 3.4.5.

To establish the immunophenotype of the neoplasms, immunohistochemistry was performed for estrogen (ER, ready-to-use, Ep1, Dako[®]) and progesterone (PR, 1:50, hpra2, Thermo Fisher[®]) receptors, cell proliferation factor Ki-67 (1:50, mib 1, Dako[®]) and epidermal growth factor 2 (HER-2, 1:200, c erb 2, Thermo Fisher[®]) in all selected samples. Immunolabeling for cyclooxygenase 2 (Cox-2, ready-to-use, Sp21, Invitrogen[®]) was also performed on 59 samples. The antibodies are well standardized for the technique used in the laboratory routine and the antigenic specificity has already been tested, as shown in previous publications (Nakagaki et al. 2022; Vieira et al. 2022, Flecher et al. 2024). Due to wear of the paraffin block during the study, two cases lacked enough material for this analysis. For the

evaluation of ER and PR labeling, a scoring system considering nuclear labeling according to intensity (negative: <1%, +: 1-25% of labeled cells; ++: 26-50% of labeled cells, + ++: 51-75%; + +++: >75% of labeled cells) (Hammond et al. 2010) was selected. Ki-67 was accounted for by nuclear labeling in 500 cells, in the Image J[®] imaging system, through photomicrographs made in a 40x (0.096 mm²) objective hot spot, and the cutoff point $\geq 20\%$ of labeled cells was used for classification in high and low proliferation (Nunes et al. 2022). HER-2 expression followed the guidelines of membrane marking score, considering scores 0, 1+ and 2+ as negative, and 3+ as positive (Wolff et al. 2013). Then, the neoplasms were categorized into Luminal A (ER and/or PR-positive, HER2 negative, and Ki-67 <20%); Luminal B HER2 negative (ER and/or PR-positive, HER2 negative, and Ki-67 $\geq 20\%$), Luminal B HER2 positive (ER and/or PR-positive, HER2 positive, and any Ki-67), HER2 overexpressed (ER and PR-negative, and HER2 positive), and Triple Negative (TN) (ER, PR, and HER2 negative) (Nunes et al. 2022, Flecher et al., 2024). Normal mammary gland was used in positive control, and a buffer solution was used as negative control, instead of the primary antibody. The Cox-2 assessment was based on a semi-quantitative evaluation of labeled tumor cells in the cytoplasm and on a distribution (0= absent, 1= less than 10% of cells labeled, 2= 10 to 30% of cells labeled, 3= 31 to 60% of cells labeled, 4= more than 61% of cells labeled) and intensity score (0= absent, 1= weak (+) labeling, 2= moderate (++) labeling, and 3= strong (+++) labeling. The final score is established by multiplying the distribution by intensity (Lavallo et al. 2009).

Anti-CD3 (1:200, Dako[®], A052) antibodies were used for the identification of T cells, while anti-CD79 antibodies were used (1:400, Biolegend[®], HM47) for identification of B cells. Normal dog lymph nodes were used as positive and negative control. A buffer solution was used for negative control, instead of the primary antibody. The negative control was done following the same technique described in the determination of immunophenotypes.

Characterization of the infiltrate in histological slides and immunohistochemistry

Images of eight *hot spots* at 600x (60x objective and 10x ocular) were captured from the H&E slides and contained a fragment of each neoplasm for quantification of TILs and TANs in the peri and intratumoral areas, separately. For the quantification of T and B cells labeled for CD3⁺ and CD79⁺, images of eight fields were captured at 400x, following the same capture methodology made on the H&E slides. Photomicrographs were taken by a Spot Insight Color digital camera adapted on the Olympus BX-40 microscope with the aid of SPOT capture software-version 3.4.5. (Estrela-Lima et al. 2010).

The identification of inflammatory cells (neutrophils and lymphocytes) on the histological slides was based on the morphological aspect of each cell. In the slides submitted to immunolabeling, lymphocytes were positive when the label was identified in the cytoplasm in a diffuse or granular way (nuclear labeling was not considered).

Inflammatory cells (lymphocytes, their subtypes and neutrophils) were counted separately in the peritumoral area (in the capsule or connective tissue around the neoplasm) and intratumoral area (in the stroma between the neoplastic lobes and in direct contact with the tumor cells) in the eight photographed fields of each sample. Finally, the number of each type of inflammatory cell, in the respective areas, was summed separately for each neoplasm, and the cutoff point to characterize how high and low intensity of inflammatory infiltrate was based on the median value of each cell. From the total number of cells, neutrophil-lymphocyte (N/L) and CD79⁺/CD3⁺ lymphocytes ratios were taken, and the median also served as a cutoff point to separate the samples with high and low expression of inflammatory cells.

All procedures performed in this studied followed the ethical principles for the use of animals in experimentation. The project was approved by the Ethics Committee on the use of animals (CEUA-UFMG, no. 11/2017).

Statistics

GraphPad Prism software version 8.2 was used for the statistical analysis. Continuous variables (mitotic count and Ki-67 expression) had their normality verified by the Kolmogorov-Smirnov's test, and the association with the intensity of inflammatory infiltrate was made by the Mann Whitney Test. For comparison of inflammatory cell infiltrate between different areas on the same tumor, the Wilcoxon test was used. Fisher's exact test was used for the association between qualitative variables (histological types, immunophenotypes, tumor size, presence of lymph node metastasis and cox-2 expression) and intensity of inflammation. In all cases, $p < 0.05$ was considered significant. The analysis of overall and specific survival was made by Kaplan Meier curves and the Long-rank test provided the comparison between curves. For the analysis of survival in relation to inflammatory intensity, the intensity of each cell type (high and low) was compared between peritumoral and intratumoral areas. Survival was also associated with N/L and CD3⁺/CD79⁺ lymphocytes ratios. The Cox regression model was performed in Jamovi version 2.3.28 for univariate and multivariate analysis, considering variables whose significance was previously established in the survival test by Kaplan Meier.

Results

Sixty-one neoplasms with solid arrangement were used. Six (9.8%) malignant myoepitheliomas, 8 (13.1%) solid papillary carcinomas, 9 (14.8%) carcinomas with solid pattern, 19 (31.1%) basaloid carcinomas, and 19 (31.1%) malignant adenomyoepitheliomas. As for immunophenotypes, 13 (21.3%) were luminal A, 42 (68.9%) were luminal B HER-2 negative and 6 (9.8%) were luminal B HER-2 positive. None of the neoplasms were TN or HER-2 overexpressed.

The animals in the study ranged in age from 3 to 19 years, with a mean age of 11 years (standard deviation 3.3). The majority were mixed-breed animals (18/61, 29.5%), followed by poodles (16/61, 26.23%) and yorkshire terriers (4/61, 6.56%), german shepherd and shih tzu

with the same amount (3/61, 4.92%). Other breeds in smaller numbers included, Dachshunds, French Bulldog, Akita, Maltes, Rottweilers and Beagle.

In quantitative analysis, the total number of lymphocytes was significantly higher than that of neutrophils ($p=0.003$). Most neoplasms 44/61 (72.1%) presented greater number of lymphocytes in the peritumoral area than in the intratumoral area ($p=0.0001$). This was also observed with neutrophils, with infiltration in the peritumoral area being greater than in the intratumoral area in 45/61 samples (73.8%) ($p=0.02$).

In the count of lymphocyte subtypes after immunostaining for $CD3^+$ and $CD79^+$, 57 of 61 (93.4%) neoplastic samples showed greater infiltration of total $CD3^+$ cells when compared to $CD79^+$ cells, which was higher in only 4/61(6.6%) samples ($p=0.0001$). Regarding the location of the infiltrate of $CD3^+$ cells, 37/61 (60.7%) neoplasms presented a higher amount of those in the intratumoral area compared to the peritumoral area, but no significant difference was seen ($p=0.20$). Meanwhile, $CD79^+$ cells were significantly greater in the peritumoral area in 47/61 (77.3%) neoplasms than in the intratumoral area ($p=0.02$).

Median and standard deviation values of each cell type are in Table 1. Tables 2 and 3 contain the morphological and immunophenotypic characteristics of the infiltrate in each subtype of neoplasms.

Table 1. Counts of lymphocyte and neutrophil (H&E) and $CD3^+$ and $CD79^+$ cells (immunohistochemistry) in canine mammary neoplasms with solid arrangement (n = 61)

	L PT	L IT	N PT	N IT	CD3 ⁺ PT	CD3 ⁺ IT	CD79 ⁺ PT	CD79 ⁺ IT
Mean	305	185	13.9	11.0	258	302	122	84.2
Median	221	100	8	5	202	215	73	24
Standard deviation	295	301	18.1	22.9	214	287	160	130

n: sample number; L: Lymphocytes; PT: peritumoral; IT: intratumoral

Table 2. Morphological and immunophenotypic characteristics and frequency of infiltration intensity of lymphocytes and neutrophils in canine mammary neoplasms with solid arrangement (n = 61). Fisher's exact test.

Variables	Lymphocytes						Neutrophils					
	PT		p	IT		p	PT		p	IT		p
	Low	High		Low	High		Low	High		Low	High	
Histological types												
CSP	3 (33.3%)	6 (66.7%)	0.68	3 (33.3%)	6 (66.7%)	0.02*	2 (22.2%)	7 (77.8%)	0.43	4 (44.4%)	5 (55.6%)	0.68
SPC	5 (62.5%)	3 (37.5%)		2 (25%)	6 (75%)		4 (50%)	4 (50%)		3 (37.5%)	5 (62.5%)	
BC	11 (57.9%)	8 (42.1%)		15 (78.9%)	4 (21.1%)		9 (47.4%)	10 (52.6%)		12 (63.2%)	7 (36.8%)	
MM	3 (50%)	3 (50%)		2 (33.3%)	4 (66.7%)		4 (66.7%)	2 (33.3%)		3 (50%)	3 (50%)	
MAME	8 (42.1%)	31 (57.9%)		7 (37.8%)	12 (63.2%)		11 (57.9%)	8 (42.1%)		8 (42.1%)	11 (57.9%)	
Immunophenotype												
L A	8 (61.5%)	5 (38.5%)	0.47	4 (30.8%)	9 (69.2%)	0.26	9 (69.2%)	4 (30.8%)	0.1	8 (61.5%)	5 (38.5%)	0.2
L B HER-2 -	20 (47.6%)	22 (52.4%)		23 (54.8%)	19 (45.2%)		20 (47.6%)	22 (52.4%)		21 (50%)	21 (50%)	
L B HER-2 +	2 (33.3%)	4 (66.7%)		2 (33.3%)	4 (66.7%)		1 (16.7%)	5 (83.3%)		2 (33.3%)	4 (66.7%)	
Size (T)												
1 (1-3cm)	14 (56%)	11 (44%)	0.67	16 (64%)	9 (36%)	0.09	12 (48%)	13 (52%)	1.0	14 (56%)	11 (44%)	0.67
2 (3 - 5 cm)	5 (41.7%)	7 (58.3%)		4 (33.3%)	8 (66.7%)		6 (50%)	6 (50%)		5 (41.7%)	7 (58.3%)	
3 (>5 cm)	11 (45.8%)	13 (54.2%)		9 (37.5%)	15 (62.5%)		12 (50%)	12 (50%)		11 (45.8%)	13 (54.2%)	
Nodal Metastasis												
Present	7 (33.3%)	14 (66.7%)	0.1	11 (52.4%)	10 (47.6%)	0.79	6 (28.6%)	15 (71.4%)	0.05	8 (38.1%)	13 (61.9%)	0.28
Absent	23 (57.5%)	17 (42.5%)		19 (47.5%)	21 (52.5%)		23 (57.5%)	17 (42.5%)		22 (55%)	18 (45%)	
Cox-2												
negative	26 (63.4%)	15 (36.6%)	0.55	23 (54.8%)	19 (45.2%)	0.25	21 (50%)	21 (50%)	0.57	23 (54.8%)	19 (45.2%)	0.09
positive	9 (52.9%)	8 (47.1%)		6 (35.3%)	11 (64.7%)		7 (41.2%)	10 (58.8%)		5 (29.4%)	12 (70.6%)	
PT: peritumoral; IT: intratumoral; CSP: carcinoma with solid pattern; SPC:solid papillary carcinoma; BC: basaloid carcinoma; MAME: malignant adenomyoepithelioma; MME: malignant myoepithelioma L: luminal; *statistically significant when p < 0.05												

PT: peritumoral; IT: intratumoral; CSP: carcinoma with solid pattern; SPC: solid papillary carcinoma; BC: basaloid carcinoma; MAME: malignant adenomyoepithelioma; MME: malignant myoepithelioma L: luminal; *statistically significant when $p < 0.05$

Table 3. Morphological and immunophenotypic characteristics and frequency of infiltration intensity of TCD3⁺ and BCD79⁺ cells in canine mammary neoplasms with solid arrangement (n = 61). Fisher's exact test.

Variables	Lymphocytes CD3						Lymphocytes CD79					
	PT		p	IT		p	PT		p	IT		p
	Low	High		Low	High		Low	High		Low	High	
Histological types												
CSP	3 (33.3%)	6 (66.7%)	0.65	3 (33.3%)	6 (66.7%)	0.23	3 (33.3%)	6 (66.7%)	0.68	1 (11.1%)	8 (88.9%)	0.19
SPC	5 (62.5%)	3 (37.5%)		2 (25%)	6 (75%)		5 (62.5%)	3 (37.5%)		4 (50%)	4 (50%)	
BC	8 (42.1%)	11 (57.9%)		10 (52.6%)	9 (47.4%)		8 (42.1%)	11 (57.9%)		11 (57.9%)	8 (42.1%)	
MM	4 (66.7%)	2 (33.3%)		5 (83.3%)	1 (16.7%)		3 (50%)	3 (50%)		2 (33.3%)	4 (66.7%)	
MAME	10 (52.6%)	9 (47.4%)		10 (52.6%)	9 (47.4%)		11 (57.9%)	8 (42.1%)		9 (47.9%)	10 (52.6%)	
Immunophenotype												
L A	9 (69.2%)	4 (30.8%)	0.28	10 (76.9%)	3 (23.1%)	0.04*	7 (53.8%)	6 (46.2%)	0.92	7 (53.8%)	6 (46.2%)	0.71
L B HER-2 -	18 (42.9%)	24 (57.1%)		19 (45.2%)	23 (54.8%)		20 (47.6%)	22 (52.4%)		18 (42.9%)	24 (57.1%)	
L B HER-2 +	3 (30%)	3 (50%)		1 (16.7%)	5 (83.3%)		3 (50%)	3 (50%)		2 (33.3%)	4 (66.7%)	
Size (T)												
1 (1-3cm)	12 (48%)	13 (52%)	0.84	11 (44%)	14 (56%)	0.56	14 (56%)	11 (44%)	0.67	13 (52%)	12 (48%)	0.31
2 (3 - 5 cm)	5 (41.7%)	7 (58.3%)		5 (41.7%)	7 (58.3%)		5 (41.7%)	7 (58.3%)		3 (25%)	9 (75%)	
3 (>5 cm)	13 (54.2%)	11 (45.8%)		14 (58.3%)	10 (41.7%)		11 (45.8%)	13 (54.2%)		11 (45.8%)	13 (54.2%)	
Nodal Metastasis												
Present	11 (52.4%)	10 (47.6%)	0.79	7 (33.3%)	14 (66.7%)	0.1	21 (52.5%)	19 (47.5%)	0.59	5 (23.8%)	16 (76.2%)	0.03*
Absent	19 (47.5%)	21 (52.5%)		23 (57.5%)	17 (42.5%)		9 (42.9%)	12 (57.1%)		22 (55%)	18 (45%)	
Cox-2												
negative	19 (45.2%)	23 (54.8%)	0.25	21 (50%)	21 (50%)	1.0	19 (45.2%)	23 (54.8%)	0.25	22 (52.4%)	20 (47.6%)	0.15
positive	11 (64.7%)	6 (35.3%)		9 (52.9%)	8 (47.1%)		11 (64.7%)	6 (35.3%)		5 (29.4%)	12 (70.6%)	
PT: peritumoral; IT: intratumoral; CSP: carcinoma with solid pattern; SPC:solid papillary carcinoma; BC: basaloid carcinoma; MAME: malignant adenomyoepithelioma; MME: malignant myoepithelioma L: luminal; *statistically significant when p < 0,05												

PT: peritumoral; IT: intratumoral; CSP: carcinoma with solid pattern; SPC: solid papillary carcinoma; BC: basaloid carcinoma; MAME: malignant adenomyoepithelioma; MME: malignant myoepithelioma L: luminal; *statistically significant when $p < 0,05$

Association between inflammatory infiltrate in relation to subtypes and immunophenotypes of solid neoplasms

In the evaluation of TILs, most CSP (6/66.7%), SPC (6/75%), MME (4/66.7%) and MAME (12/63.2%) neoplasms showed significantly higher intensity of TILs in the intratumoral

area when compared to BC tumors, in which only 4/21.1% neoplasms of this type presented high intensity of TILs ($p=0.02$). In the peritumoral area, no difference in the intensity of the lymphocyte infiltrate was observed between histological types (Fig.1 A and B). The intensity of TANs, TCD3⁺ and BCD79⁺ cells in the peri and intratumoral areas showed no significant differences between histological types. (Tab. 1 and 2). Fig. 2 shows the intensity of cellular infiltration between basaloid carcinoma and carcinoma with solid pattern.

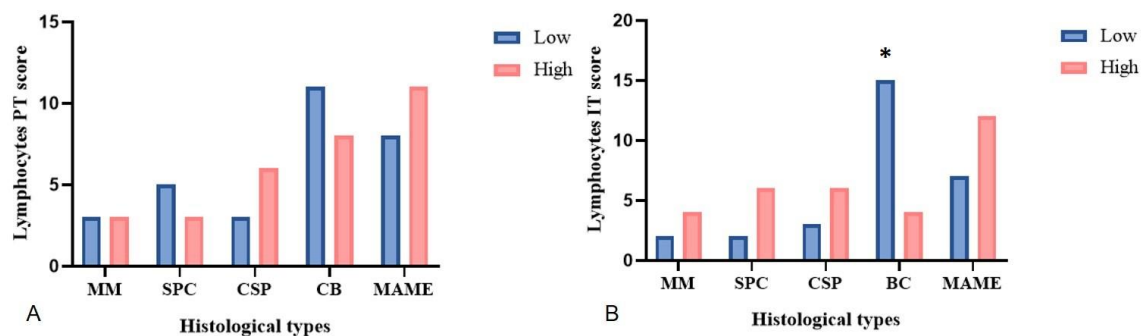


Fig. 1 Comparison of the intensity of TILs in relation to the types of neoplasms with solid arrangement of the canine mammary gland, by Fisher's exact test. A. Intensity of infiltration of peritumoral TILs between histological types. B. Predominance of neoplasms with high intensity of intratumoral TILs in MME, SPC, CSP and MAME, except BC ($p=0.02$). MME: Malignant myoepithelioma; SPC: Solid papillary carcinoma; CSP: Carcinoma with solid pattern; BC: Basaloid carcinoma; MAME: Malignant adenomyoepithelioma.

Neoplasms with luminal B immunophenotypes (HER-2 negative or positive) showed higher intensity of intratumoral TCD3⁺ lymphocytes when compared to Luminal A neoplasms ($p=0.04$). Three out of thirteen (23.1%) luminal A neoplasms presented high infiltration of intratumoral TCD3⁺ lymphocytes, while Luminal B HER-2 negative or HER-2 positive presented higher percentage of neoplasms with high intensity of this cell type, being 23/42 (54.8%) and 5/6 (83.3%), respectively. The intensity of infiltration of CD79⁺ cells (Fig.3), as TILs and TANs, and their respective locations, showed no significant association with

immunophenotypes.

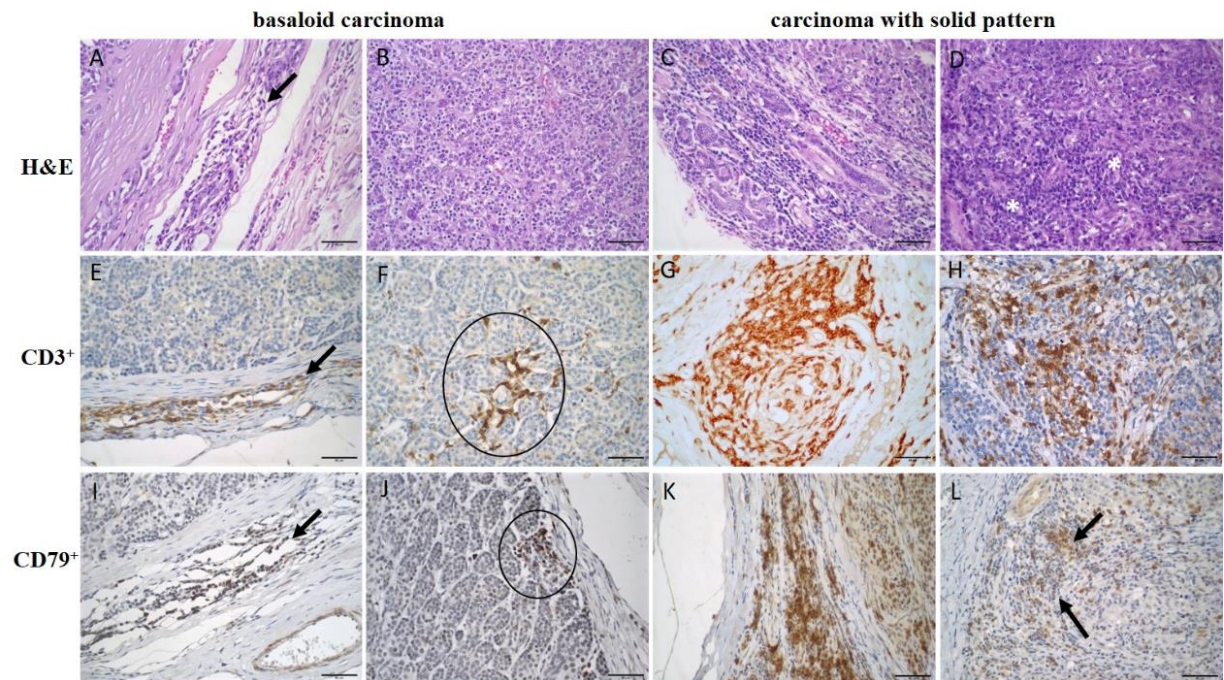


Fig. 2 Intensity of inflammatory infiltrate in basaloid carcinoma compared to carcinoma with solid pattern. A to D. TILs on slides stained in H&E (600x); A. Low amount of peritumoral TIL in BC (arrow); B. intratumoral TIL in BC; C. peritumoral TIL high in CSP; D. high intratumoral TIL and forming aggregates in CSP (white asterisk); E to H. Infiltrate of TCD3⁺ cells in immunohistochemistry (400x); E. Low amount of peritumoral lymphocytes in BC (arrow); F. Small aggregates of intratumoral lymphocytes in contact with neoplastic cells in BC (circle); G. High infiltration of peritumoral lymphocytes in CSP; H. High infiltration of intratumoral lymphocytes in CSP; I to L: Infiltrate of TCD79⁺ cells in immunohistochemistry (400x). I. Low infiltration of peritumoral lymphocytes in the BC (arrow); J. Low infiltration of intratumoral lymphocytes in the BC (circle); K. High infiltration of peritumoral lymphocytes in the CSP; l. High infiltration of intratumoral lymphocytes in the CSP (arrow).

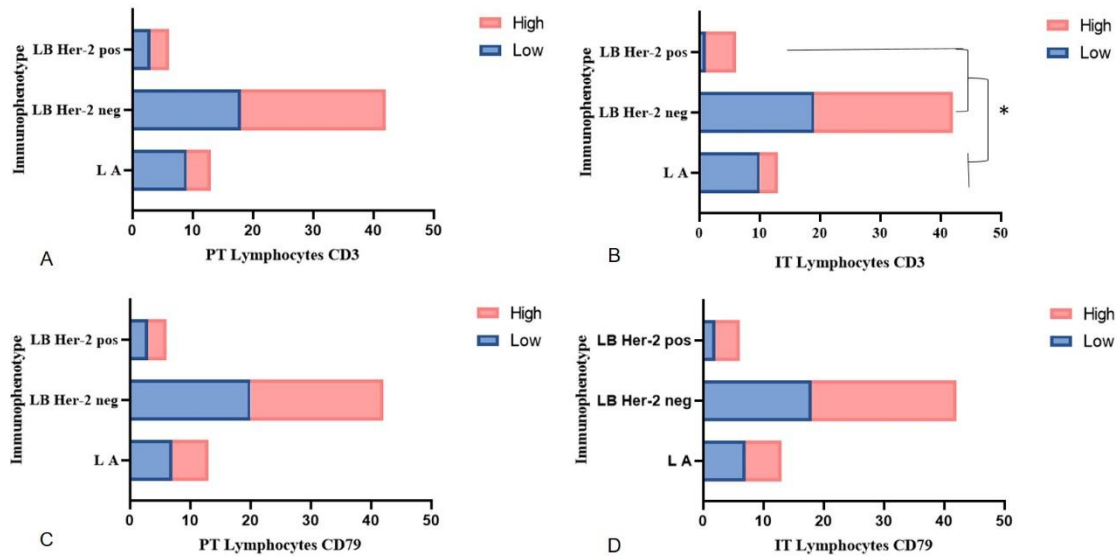


Fig. 3 Intensity of infiltration of TCD3⁺ and BCD79⁺ relative to Luminal A, Luminal B HER-2 negative and Luminal B HER-2 positive immunophenotypes in solid arrangement neoplasms, Fisher's exact test. (A) Intensity of peritumoral TCD3⁺ cells (p=0.28); (B) Luminal B neoplasms have higher intensity of intratumoral TCD3⁺ cells compared to Luminal A (p=0.04*); C. Intensity of peritumoral BCD79⁺ cell infiltration (p=0.92); D. Intensity of intratumoral BCD79⁺ cells (p=0.71). LA: Luminal A; LB: Luminal B.

Association between proliferative rate, presence of lymph node metastases and inflammatory infiltrate.

Twenty-one bitches (34.4%) had lymph node metastases. Of these, 16 (76.2%) had high intensity of intratumoral BCD79⁺ lymphocytes infiltrate (p=0.03). On the other hand, the intensity of this peritumoral cell was not associated with the presence of metastasis (p=0.59) (Fig. 4). We also observed that 15 (71.9%) of the 21 neoplasms that had metastasized to lymph node presented an intensity of TANs above the median in the peritumoral area, but this was not significant (p=0.05). For TILs and TCD3⁺ cells, no significant difference was observed between intensity and location of inflammation and lymph node status.

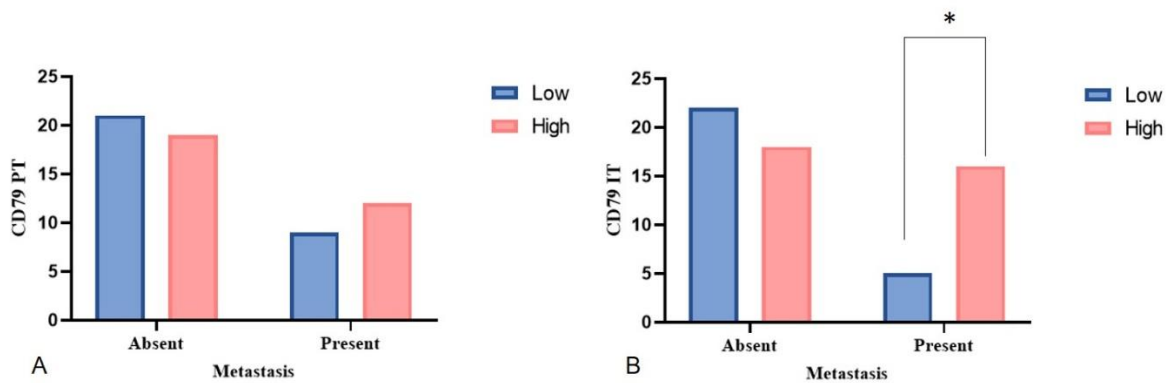


Fig. 4 Intensity of inflammatory infiltrate in relation to the presence of metastases in regional lymph nodes. Fisher's Exact Test. (A) Infiltration of peritumoral BCD79⁺ cells showed no difference between neoplasms with or without lymph node metastases ($p=0.59$). (B) Infiltration of intratumoral BCD79⁺ cells was high in bitches that had metastasis compared to those that did not ($p=0.02$). *statistically significant

Neoplasms with high density of intratumoral TILs had a higher mitotic count with a median of 20 mitosis figures (2.37mm^2), while neoplasm with low density of TILs had a median of 14 mitosis figures ($p=0.03$). However, the density of peritumoral TILs was not significantly related to mitotic count ($p=0.05$). High density of peritumoral TCD3⁺ and BCD79⁺ cells was also associated with higher mitotic count. Neoplasms with high infiltration of peritumoral TCD3⁺ and BCD79⁺ lymphocytes presented a median of 21 mitosis figures, while those with low infiltration presented 13 mitosis figures ($p=0.006$) for both cell types. Intratumoral infiltration was only significant in relation to mitotic count for BCD79⁺ cells, in which neoplasms with high intensity had a median of 19 mitosis figures, and those with low infiltration had 12 mitosis figures ($p=0.02$). The intensities of peri ($p=0.9$) and intratumoral ($p=0.7$) TANs were not associated with mitotic counting.

When evaluating the intensity of inflammation with Ki-67 immunostaining, neoplasms with high TILs ($p=0.02$) and high infiltration of TCD3⁺ ($p=0.007$) and BCD79⁺ cells ($p=0.02$) in the peritumoral area were associated with a higher percentage of immunostaining (Fig. 5). Neoplasms with high intensity of TILs, TCD3⁺ and BCD79⁺ cells had the same median of Ki-67 labeling (34%), and neoplasms with low intensity of those cells had a median of 26%, 25%

and 26% of immunostaining, respectively. The remaining locations of TILs, CD3⁺ and CD79⁺ cells, as well as TANs, were not associated with the percentage of Ki-67 labeling.

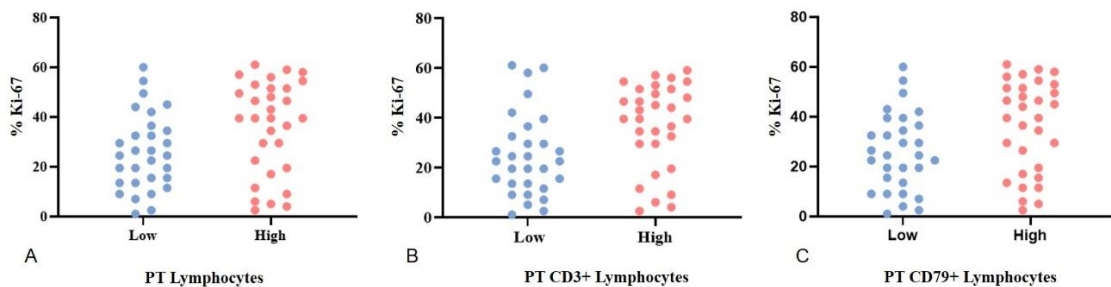


Fig. 5 Association of peritumoral inflammation intensity with immunostaining for Ki-67 in neoplasms with solid arrangement. Mann-Whitney's test. (A) High intensity of TILs ($p=0.02$), high intensity of TCD3⁺ cells ($p=0.007$) and BCD79⁺ cells ($p=0.02$) are associated with higher percentage of Ki-67.

Association between tumor size and Cox-2 immunostaining with inflammatory infiltrate.

The results are detailed in tables 1 and 2.

Among the neoplasms evaluated ($n=61$), 25 (41%) were classified as T1, 12 (19.7%) as T2 and 24 (39.3%) as T3. No association of tumor size with infiltrate intensity of TILs, TANs, TCD3⁺ and BCD79⁺ was observed.

Of the 59 neoplasms evaluated for Cox-2, only 17 (28.8%) presented positive immunostaining; 16 presented scores up to 3 and one presented score 6. No statistical difference was seen between peri and intratumoral inflammations and Cox-2 positivity.

Association between inflammatory infiltrate and survival time

Information on overall survival was available for 47/61 (77%) bitches, of which 35 (74%) died and 12 (26%) were alive at the end of the study. Of the 35 dogs that died, 22 (63%) died from causes related to the mammary tumor. The median overall survival was 365 days (95% CI, 283 to 630 days) and the specific survival was 670 days (95% CI, with a minimum of 365).

Bitches who had neoplasms with high intensity of intratumoral TANs had shorter median overall survival time than those with low infiltration of such cells ($p=0.04$), being 210 and 425 days of survival, respectively. On the other hand, infiltration with peritumoral TANs ($p=0.2$)

had no influence on survival. Also, overall survival was not associated with TILs in the peri (p=0.14) and intratumoral (p=0.10) areas, infiltration of peri (p=0.48) and intratumoral (p=0.05) TCD3⁺ cells and infiltration of peri (p=0.17) and intratumoral (p=0.11) BCD79⁺ cells (Table 4).

In the specific survival evaluation, 50% of bitches presenting neoplasms with high intensity of intratumoral TILs had died in 395 days, and 64% of those with low intensity of TILs were still alive after 1,000 days (p=0.02). That was also similar to what was observed in the evaluation of TCD3⁺ cells, whose patients with high intratumoral intensity a median survival time of 365 days, and 61% those with low intratumoral intensity were still alive after 1,000 days (p=0.01). The intensity of peritumoral TILs (p=0.53) and TCD3⁺ (p=0.05) (p=0.11), peri (p=0.11) and intratumoral (p=0.06) BCD79⁺ cells, as well as peri (p=0.48) and intratumoral TANs (p=0.28) was not associated with dog survival (Fig.6; table 4).

Table 4. Median survival time of bitches with neoplasms with solid arrangement and association with the intensity of lymphocyte infiltrate, neutrophils, TCD3⁺ and TCD79⁺ cells.

Cell infiltration	Median overall survival (days)					
	Peritumoral			Intratumoral		
	<i>Low</i>	<i>High</i>	<i>P</i>	<i>Low</i>	<i>High</i>	<i>P</i>
Lymphocytes	455	324	0.14	425	321	0.1
Neutrophils	395	365	0.2	425	210	0.04*
CD3 Lymphocytes	395	341	0.48	425	360	0.05
CD79 Lymphocytes	500	365	0.17	575	318	0.11
	Median specific survival (days)					
Lymphocytes	670	720	0.53	-----	395	0.02*
Neutrophils	670	575	0.48	1035	575	0.28
CD3 Lymphocytes	670	720	0.5	-----	365	0.01*
CD79 Lymphocytes	-----	395	0.11	-----	395	0.06

*statistically significant when $p < 0.05$. Long rank teste. ----- more than 50% of animals were still alive at the end of the study.

The N/L ratio and CD3+/CD79 cells were also unrelated to the overall ($p=0.54$; $p=0.53$) and specific ($p=0.81$, $p=0.57$) survival time of bitches.

In the specific survival analysis by Cox regression, the univariate analysis showed that high intensity of intratumoral TILs increases the risk of death related to the neoplasm. The high presence of TILs is associated with a risk ratio of 2.76 times ($p=0.03$, Confidence Interval, CI, 95%= 1.08-7.07), and the high infiltration of intratumoral TCD3⁺ cells increased the risk of death related to the neoplasm by 3 times ($p=0.02$, CI:1.19-7.8). Bitches with metastases to regional lymph nodes also had risk of death 6.5 (CI: 2.61-16.4) times higher. In the multivariate analysis, the only variable that remained associated with the risk of death was the presence of metastasis in lymph nodes, being responsible for a three-fold increase in the risk of death from the disease ($p<0.001$). (Table 5)

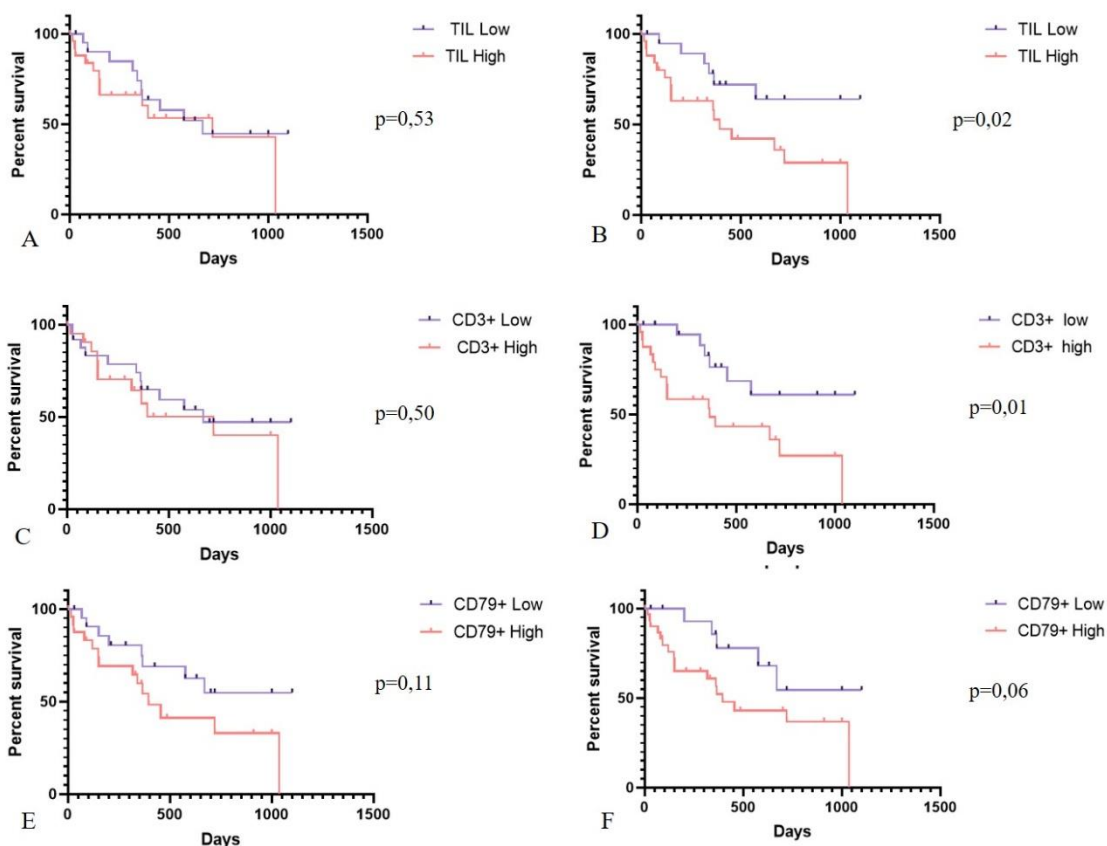


Fig. 6 Specific survival of bitches with mammary gland neoplasms with solid arrangement in relation to the presence of TILs, TCD3⁺ and BCD79⁺ cells. Kaplan-meier A. Infiltrate of peritumoral TILs, B. Infiltrate of intratumoral TILs. C. Infiltrate of peritumoral TCD3⁺ cells, D. intratumoral TCD3⁺ cells; E. Infiltrate of peritumoral B CD79⁺ cells; D. intratumoral CD79⁺ cells.

Table 5. Univariate and multivariate analyses of specific survival (Cox model) in relation to intratumoral TIL and CD3⁺ in bitches with neoplasms with solid arrangement.

<i>Variables</i>		HR	95% CI	p-value
<i>Univariate analyses</i>				
Metastasis	Absent			
	Present	6.54	2.61-16.4	<0.001
IT Lymphocytes	Low			
	High	2.76	1.08-7.07	0.03
IT CD3 Lymphocytes	Low			
	High	3.05	1.19-7.80	0.02
<i>Multivariate analyses</i>				
Metastasis	Absent			
	Present	3.23	1.09-9.57	0.03
IT Lymphocytes	Low			
	High	0.73	0,22-2.39	0,6
IT CD3 Lymphocytes	Low			
	High	0.82	0.25-2.72	0.7

HR, hazard ratio, CI, Confidence interval, , IT: intratumoral; TILs: tumor-infiltrating lymphocytes.

*statistically significant when $p < 0,05$.

Discussion

In canine neoplasms with solid arrangement, the characteristic of tumor infiltrating inflammatory cells were associated with survival time and some prognostic factors. Increase in the inflammatory infiltrate of lymphocytes and their subtypes was associated with some poor prognostic factors such as higher mitotic count, Ki-67 expression and higher frequency of metastasis to lymph nodes.

Bitches presenting neoplasms with solid arrangement with high intensity of intratumoral TILs and TCD3⁺ cells had significantly lower median for specific survival time when compared to bitches presenting neoplasms with low intensity of intratumoral TILs and CD3⁺ cells. This is in agreement with what is reported in women who also have luminal neoplasms, leading to an association of high number of TILs with shorter survival time (Gao et al. 2020). This is believed to be related to the type of lymphocytes present, since mammary neoplasms with luminal immunophenotype have a lower percentage of TCD8⁺ cells when compared to TN neoplasms (Mahmoud et al. 2011). TCD8⁺ cells are cells involved in the anti-tumor response

mechanism, leading to neoplastic cell lysis. In contrast, other types of T cells that favor cancer progression and shorten survival may be present, such as regulatory T cells (such as Foxp3) and CD4 helper 2 cells (Huang et al. 2015; Koletsa et al, 2020). A limitation of this study was not identifying T lymphocyte subtypes such as CD4⁺ and CD8⁺, or Foxp3. However, we suggest the presence of a predominant immunosuppression and tolerance response in the tumor microenvironment.

The neoplasms with solid arrangement evaluated were MME (9.8%), SPC (13.1%), CSP (14.8%), BC (31.1%) and MAME (31.1%). We had few neoplasms diagnosed as MME and SPC, which may be a limitation regarding the representativeness of the various histological types of these neoplasms. However, some of them are infrequent, as seen in a study reclassifying 72 cases of solid carcinomas in female dogs, where MME represented only 15% of these neoplasms (Yoshimura et al. 2014).

There was a significant statistical predominance of lymphocytes in relation to neutrophils in all samples, corroborating other studies that evaluated the inflammatory infiltrate in several histological types of mammary neoplasms in bitches (Estrela-Lima et al. 2010; Souza et al. 2018). Lymphocytes are the most studied cells in the tumor microenvironment of several canine neoplasms, such as oral and cutaneous melanomas, (Gregório et al. 2016; Porcellato et al. 2019) urothelial bladder cell carcinoma (Inoue et al. 2017) and mammary cancer (Estrela-Lima et al. 2010; Kim et al. 2013; Souza et al. 2018). However, few authors specify the areas where this inflammation is located (Carvalho et al. 2011; Muscatello et al. 2022; Vieira et al. 2022).

In canine mammary neoplasms with solid arrangement, the stroma is often scarce among neoplastic nests, making it difficult to assess stromal lymphocytic inflammation as it is done in women. Therefore, we evaluated peritumoral and intratumoral inflammation as was already done in other studies in veterinary medicine (Estrela-lima et al. 2010; Vieira et al. 2022). In women's breast cancer, the evaluation of TILs focusses on the stromal area between neoplastic

nests, and the percentage of the area occupied by the inflammation is used in prognostic and predictive analysis (Salgado et al. 2015).

In histology, we identified that most of the evaluated neoplasms show greater amount of infiltration of TILs in the peritumoral area (72.1%) in relation to the intratumoral area ($p=0,0001$). And when evaluating the lymphocyte subtype, the majority of neoplasms (93.4%) showed a significantly higher amount of T lymphocytes than B lymphocytes ($p=0,0001$), similar to what has already been described in women (Gomez-Macias et al. 2020), cats (Nascimento et al. 2022) and bitches (Muscatello et al. 2022). There was no statistical difference in the amount of TCD3 lymphocytes between the peri and intratumoral areas in the samples, but CD79 B lymphocytes predominated in the peritumoral area ($p=0,02$). Similar to what is seen in breast cancer in women, TCD3⁺ cells show reasonable homogeneity in samples, and may be around and inside the nests of tumor cells, while CD20⁺ cell, a marker used for B cells in women, is seen in less quantity and often forming peritumoral aggregates (Mani et al. 2016). The formation of peritumoral lymphoid aggregates, which consist predominantly of B cells, is reported in *in situ* and *invasive* ductal carcinoma of women, indicating a host's humoral immune response secondary to the neoplastic cell proliferation (Ishigami et al. 2019).

BCs are characterized by compact cell nest formations with cells presenting more hyperchromatic nuclei, especially in the periphery, where cells are organized into palisades (Nakagaki et al. 2022). This may cause greater difficulty in the visualization of lymphocytes within the tumor nests, and may have influenced in the lower intensity of intratumoral TIL that we found in this histological type compared to the others ($p=0.02$). Thus, we may infer the histological and growth patterns of the neoplasm can interfere with the quantification of TILs, as described in women's neoplasms (Salgado et al. 2015).

As for immunophenotypes, only luminal A, luminal B HER-2 negative and luminal B HER-2 positive immunophenotypes were diagnosed. In this study, neoplasms with luminal B

HER-2 positive and negative immunophenotype presented significantly higher intensity of intratumoral TCD3⁺ lymphocytes infiltration when compared to those of luminal A immunophenotype (p=0.04). The mammary cancer immunophenotype is known to influence the amount and composition of inflammatory infiltrate in the tumor microenvironment (Glajcar et al. 2019; Zhu et al. 2019). According to Bergholtz et al. (2022), canine luminal A neoplasms may present less activation of genes that produce proteins associated with immune mechanism and inflammatory stimulus than B immunophenotype neoplasms. In women, luminal A mammary neoplasms have also been described as presenting lower presence of TILs than luminal B HER-2 negative neoplasms (Criscitiello et al. 2020). Similarly, TN and HER-2 overexpressed neoplasms also incite greater chemotaxis, both of lymphocytes (Lee et al. 2015; Wang et al. 2016; Koletsa et al. 2020) and neutrophils (Soto-Perez-de-Celis et al. 2017), due to greater genomic instability.

During the inflammatory process, several growth factors and cytokines that activate transcription factors are produced, such as NF- κ B and STAT-3, which favor cell proliferation (Shrihari et al. 2017). This is in agreement with what we observed, i.e., high intensity of intra and peritumoral lymphocyte inflammation was observed in neoplasms with higher mitotic count and/or higher immunostaining of Ki-67. In the present study, high mitotic count was associated with high intensity of intratumoral TIL (p=0.03) and CD79⁺ (p=0.02). In addition, both mitotic count and Ki-67 immunostaining were proportionally related to the intensity of peritumoral CD3⁺ and CD79⁺ cells. As cells proliferate, they can expose new antigens and induce a greater inflammatory response, primarily by cells of the innate immune system, such as TCD8⁺ and NK lymphocytes.

Metastasis to regional lymph node was significantly associated with high intensity of intratumoral lymphocytes. This finding corroborated other studies of canine mammary tumors that describe a higher percentage of CD79⁺ cells infiltrating carcinoma in mixed tumor of

animals presenting metastasis to lymph nodes (Estrela-Lima et al. 2010) and greater peritumoral lymphatic invasion, in several types of mammary carcinomas, when compared to tumors with low infiltration of such cells (Kim et al. 2013). In canine melanoma, lymph node metastasis is also related to a moderate amount of intratumoral B cells in the primary neoplasm (Porcellato et al. 2019). In both women and in experimental mouse models, increased infiltration of B cells was detected in the microenvironment of neoplasms that metastasized to lymph nodes (Gu et al. 2019; Ishigami et al. 2019), and many of those lymphocytes were regulatory B cells, responsible for producing cytokines that reduce the anti-tumor function of T cells via secretion of IL-10 (Tao et al. 2015; Ishigami et al. 2019). In experimental models, such increase in tumor B cells is associated with the appearance of stromal cells in the lymph node and increase of transcription genes related to important signaling pathways in pre-metastatic niches in lymph nodes (Gu et al. 2019). Even though CD79⁺ B lymphocytes were associated with lymph node metastasis in the bitches of the present study, the high infiltration of those did not influence the overall and specific survival time. However, we observed that the presence of lymph node metastasis increased the risk of death from the neoplasm by six and three times in a univariate and multivariate analysis, respectively, which makes it an independent prognostic factor. Metastasis to lymph nodes was also a factor that increased the risk of cancer-related death in bitches in other studies (Estrela-Lima et al. 2010; Nunes et al. 2022).

Although we did not observe a significant association of neutrophils with the presence of metastasis to lymph nodes, 71,4% of the neoplasms that metastasized had high intensity of peritumoral TANs ($p=0.05$). In addition, intratumoral neutrophil infiltration generated an important difference in the overall survival time of bitches; those with low intratumoral intensity had median survival time of 425 days, while for those with high intensity of neutrophil infiltrate had median survival time of 210 days ($p=0.04$). In human medicine, few breast cancer articles make this association. One of them describes shorter survival time in patients with high

density of neutrophil infiltrate, but without specifying the location (Kwantwi et al. 2021). To date, no other study associating tumor neutrophil infiltration with the survival of bitches with mammary neoplasms is known.

Some studies associate a larger tumor size with greater presence of TILs in women's luminal neoplasms (Glajcar et al. 2019; Murazawa et al. 2023), but we did not observe a significant association of this variable with TILs, T cells (CD3⁺), B cells (CD79⁺) and neutrophils.

A higher Cox-2 score was associated with higher tumor inflammatory infiltrate in mammary neoplasms (Carvalho et al. 2016) and canine melanoma (Gregório et al. 2016). However, that finding was not observed in the present study. This may have occurred because we had only 28.8% of neoplasms marked for Cox-2, and most of them presented low scores. Cyclooxygenase is probably not an important factor for inflammatory stimulation in neoplasms with solid arrangement, however further studies should be conducted to confirm this hypothesis.

The blood neutrophil/lymphocyte ratio has been performed in patients with mammary carcinomas and is indicated as a prognostic factor in studies of cats (Petrucci et al. 2020). In bitches, a blood ratio >5 N/L before surgery is associated with a worse prognosis (Uribe-Querol et al. 2023). Here we used the cell count in the tumor microenvironment to associate the ratio of N/L and CD79⁺/CD3⁺ with survival, but it was not significant.

In a study of mammary neoplasms in bitches, the animals presenting neoplasms with greater lymphocyte infiltration (>600 lymphocytes/8 fields) had lower survival time (Estrela-Lima et al. 2010) and this was confirmed by the multivariate cox regression analysis (Estrela-Lima et al, 2010). In the univariate analysis, we observed the high intensity of intratumoral TILs and intratumoral T lymphocytes increased the risk of death from the neoplasm by 2.7 and three times, respectively, but this was not confirmed by the multivariate analysis.

Conclusions

In our study, we observed that neoplasms with solid arrangement show greater predominance of lymphocytes than neutrophils, and mainly TCD3⁺ cells. TCD3⁺ cells predominated in the intratumoral area, while BCD79⁺ cells predominated in the peritumoral area.

The infiltration of lymphocytes and their location in canine mammary neoplasms, evaluated here, are associated with some prognostic factors in bitches. Nodal metastasis was associated with high infiltration of intratumoral CD79⁺ cells, and shorter survival time was associated with high intensity of TILs and intratumoral T CD3⁺ cells. Thus, lymphocyte infiltration, especially the intratumoral infiltration, may be an important feature in cancer progression, inciting a pro-tumor and regulatory response of the anti-tumor response and influencing the survival of bitches with mammary neoplasms with solid arrangement.

We know that, in the laboratory routine, the quantitative evaluation of inflammatory cells in the tumor microenvironment is difficult to be carried out. However, the analysis of the intensity of peri and intratumoral inflammation from a cutoff point can be performed in hematoxylin and eosine stains and associated with morphological characteristics to gather possible prognostic information.

Acknowledgments

We thank the tutors and veterinarians for providing the necessary information to carry out this study and universities that provided slides and paraffin blocks to carry out the research.

Authors' contributions

M. C. F. – Conceptualization, Methodology, Investigation, Writing - Review & Editing, Writing - Original Draft, Visualization, Project administration. M.P.R - Investigation, Writing. D. B.- Formal analysis, Writing - Review & Editing; K. Y. R. N. - Conceptualization,

Methodology, , Project administration; G. D. C. - Conceptualization, Methodology, Investigation, Writing - Review & Editing, Writing - Original Draft, Funding acquisition, Project administration

Funding

This study was funded by the National Council for Scientific and Technological Development (CNPq), the Minas Gerais Research Foundation – FAPEMIG, the Minas Gerais Network for Translational Research in Immunobiologicals and Biopharmaceuticals in Cancer (REMITRIBIC, RED-00031-21) and the Coordination for Personal Improvement of Higher Education (capes), Brazil.

Ethical Approval

All procedures performed in this study were in accordance with the ethical principles for the use of animals in experimentation approved by CEUA – UFMG (No. 11/2017).

Consent to participate. All authors participated voluntarily in the research.

Consent for publication. All authors read and approved the final manuscript.

Competing interests: The authors declare that there are no competing interests

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7. CONCLUSÕES FINAIS

- As neoplasias de arranjo sólido da glândula mamária canina são predominantemente luminal B HER-2 negativo, sendo o carcinoma com padrão sólido o mais frequente nesse imunofenótipo, seguido pelo Luminal A, no qual estava presente todos os mioepiteliomas malignos. Os imunofenótipos não foram associados a nenhum fator prognóstico avaliado, a necrose, expressão de cox-2 e sobrevida global e específica.
- O grau de inflamação é baixo nessas neoplasias, apresentando principalmente grau I e II, nas localizações peri e intratumoral, e raramente grau III, que foi evidenciado apenas na região intratumoral.
- Foi observada uma predominância de linfócitos nos tumores de arranjo sólido, sendo significativamente maior que a de neutrófilos. Dentre os subtipos de linfócitos, os linfócitos T estão em maior quantidade, se localizando principalmente na região intratumoral, enquanto os linfócitos B estão mais frequentemente na região peritumoral.
- Apesar de os imunofenótipos não terem sido associados ao grau de inflamação tumoral na histologia, as neoplasias luminais B apresentaram significativamente maior intensidade de infiltração linfócitos T CD3⁺ intratumoral que as neoplasias dos outros imunofenótipos.
- Associando a inflamação aos fatores prognósticos foi identificado que a maior intensidade de linfócitos T e B peritumoral foi estatisticamente associada à maior contagem mitótica e expressão de Ki-67. Além disso, a maior graduação de inflamação na histologia peri e intratumoral e a maior intensidade de infiltrado de linfócitos B intratumoral foram associadas a metástase para linfonodo.
- Os TILs e linfócitos T CD3 + intratumoral foram inversamente proporcionais à sobrevida das cadelas com neoplasias de arranjo sólido, entretanto não são considerados fator prognóstico independente. Por outro lado, mesmo a infiltração intratumoral de linfócitos B não ser diretamente relacionada à sobrevida, essa variável está associada à presença de metástase para linfonodo, que é um o único fator prognóstico independente para o risco de óbito pela neoplasia.

- Não foram observadas associação estatística da intensidade de neutrófilos e fatores prognósticos, mas sua maior intensidade foi associada à redução da sobrevida global das cadelas.

8. CONSIDERAÇÕES FINAIS

A princípio as neoplasias de arranjo sólido começaram a ser estudadas no Laboratório de Patologia Comparada da UFMG para determinar os tipos celulares presentes e, consecutivamente, entender as diferenças comportamentais e prognósticas dessas nessa neoplasia nas cadelas. Em virtude dos diferentes tipos histológicos, presente nesse grupo neoplásico, julgou-se necessário continuar o estudo para melhor caracterização dessas neoplasias e identificar outros fatores que poderiam estar associados ao prognóstico desfavorável associado a elas.

Assim foi possível identificar que as neoplasias com menor índice proliferativo também estão associadas a imunofenótipos de melhor prognóstico, porém o imunofenótipo das neoplasias não está associado a fatores prognósticos importantes para o câncer de mama como metástase e sobrevida. Em contrapartida, a inflamação exerce efeito importante na ocorrência de metástase, atividade proliferativa e sobrevida das cadelas.

Nesse trabalho, apesar de alguns resultados apresentarem relevância nos valores absolutos, o resultado estatístico não apresentou diferença estatística ou ficou com tendência a significância. Isso provavelmente foi relacionado ao número de amostras e falta de acompanhamento de alguns desfechos dos casos. Dentre as limitações encontradas estão falta de material presente em blocos de parafina, dificuldade de alguns tipos histológicos por serem mais raros como o mioepitelioma maligno e carcinoma com padrão sólido, não envio de linfonodo para avaliação histopatológica, reduzindo o número de amostras utilizadas, e dificuldade de informações do acompanhamento dos pacientes.

Nós fizemos a identificação de linfócitos T e B pela imuno-histoquímica, entretanto, sabemos que os linfócitos T apresentam diversos subtipos que influenciam diretamente o

microambiente tumoral, e com isso a progressão da neoplasia. Por isso, buscou-se a realização das imuno-histoquímica para linfócitos T CD8⁺ e T CD4⁺, utilizando protocolos distintos, além de envio de amostra para um outro laboratório como parceria, mas não tivemos sucesso.

Por fim, esse trabalho, em concordância com outros estudos do grupo, mostrou a importância da inflamação na progressão do câncer. Por isso, julgamos necessário padronizar uma metodologia para descrever a presença do processo inflamatório e a sua localização durante a avaliação e descrição dos resultados histopatológico de câncer de mama, assim como já é feito para mulheres. Além disso, demos início a investigação da presença dos neutrófilos, e a sua associação com sobrevida das cadelas.

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ANEXOS

CRÉDITOS OBTIDOS

- 28 créditos.

Disciplinas cursadas durante o doutorado.

PERÍODO	NOME DA DISCIPLINA	CONCEITO	CRÉDITO
2020/1	Tópicos em Patologia III	A	03
2020/1	Seminários de Pesquisa em Patologia I	A	02
2020/1	Estágio Docente	A	02
2020/2	Tópicos em Patologia III	A	03
2020/2	Tópicos Esp. Clínica e Cirurgia Veterinárias	A	03
2020/2	Microscopia dos Processos Patológicos gerais	A	05
2021/1	Tópicos em Patologia I	A	01
2021/1	Tópicos em Patologia I	A	01
2021/1	Tópicos em Patologia III	A	03
2021/1	Estágio Docente	A	02
2021/2	Tópicos em Patologia III	A	03
2022/1	Elaboração de Trabalho final	-	0
2022/2	Elaboração de Trabalho final	-	0

PRODUÇÃO CIENTÍFICA DURANTE O DOUTORADO

1. Artigo aceito para publicação relacionados a tese

M.C. Flecher, D. Balabram, Y.A. Salles, F. R. Souza, A. Estrela-Lima, K.Y. R. Nakagaki, G. D. Cassali. Evaluation of immunophenotype and inflammation in canine mammary neoplasms with solid arrangement. Journal Comparative Pathology

2. Artigos Publicados não relacionados a Tese

FLECHER, M. C.; NASCIMENTO, N. T.; REYS, M. P.; GORZA, L. L.; SOUZA, M. C. C.; HORTA, R. S. Periarticular histiocytic sarcoma in a French Bulldog. *Brazilian Journal of Veterinary Pathology*. v.17, p.39 - 45, 2024.

PIMENTEL P.A.B, DA SILVA P.H.S, SENA B.V, **FLECHER M.C**, CASSALI G.D, HORTA R.D.S. The role of lymph nodes and their drainage in canine mammary gland tumours: Systematic review. *Res Vet Sci*. v.168, P.105139. 2024

GIULIANO A., LAM S.Y, FLECHER, M.C, HORTA R.S. Case report: Suspected plasmablastic lymphoma in a dog resembling the clinical presentation in humans. **Front Vet Sci**. v.16, n.10: p.1100942, 2023

SILVA, H.C.; REYS, M.P.; CASSALI, G. D.; SOUZA, F. R.; HORTA, R.S.; SENA, B.V ; BINDACO, A. ; PINTO, A.C.J. ; SOUZA, T.D. ; **FLECHER, M. C**. Secretory carcinoma of the canine mammary gland with nodal and bone metastases: case report. *OPEN VETERINARY JOURNAL*, v. 12, p. 502-507, 2022.

REYS, M.P.; **FLECHER, M.C.**; MATOS, L. S. F.; SOUZA, F. R.; CASSALI, G. D. ; SEDE, S. C. ; SOUZA, B. C. ; PEREIRA, C. M. . Solid Iridociliary Carcinoma in a Dog. *ACTA SCIENTIAE VETERINARIAE (ONLINE)*, v. 50, p. 824-828, 2022.

SOUZA, L. M.; GORZA, L. L.; RASSELE, A. C.; SENA, B. V.; ALMEIDA, I. O.; **FLECHER, M.C.** ; HORTA, R. S. . Canine Mast Cell Tumour Lomustine Sensitization by Tyrosine Kinase Inhibitors. *ACTA SCIENTIAE VETERINARIAE (ONLINE)*, v. 50, p. 839-844, 2022.

BINDACO, A.; MONTEIRO, L.N.; SÁ, M. M.; REYS, M.P.; LOBO, R.; CONTI, L.; **FLECHER, MAYRA**. Canine facial eosinophilic furunculosis in a dog. *BRAZILIAN JOURNAL OF VETERINARY PATHOLOGY*, v. 15, p. 153-156, 2022.

GORZA, L.L.; OLIVEIRA, E. C.; LOPES, C. E. B.; SILVA, E. L. F.; HIURA, E.; **FLECHER, M.C.**; SOUZA, T.D.; BRAGA, F. R.. Abortion in Captive Gray Brocket Deer (*Mazama gouazoubira*) Associated with Colloid Goiter, Hemochrosis and Necrotizing Rumenitis. *ACTA SCIENTIAE VETERINARIAE (ONLINE)*, v. 49, p. 693-698, 2021.

CAVALCANTI, E.B.O; GORZA, L.L; SENA, B.V.; SOSSAI, B.; CARRETA JUNIOR, M.; **FLECHER, M.**; MARCOLONGO-PEREIRA, C.; HORTA, R.S. Correlation of Clinical, Histopathological and Histomorphometric Features of Canine Soft Tissue Sarcomas. *BRAZILIAN JOURNAL OF VETERINARY PATHOLOGY*, v. 14, p. 151-158, 2021.

SILVA, P.H.S; DE SOUZA, M.C.C; CAVALCANTI, E.B.O; FONSECA, V.B.; SILVEIRA, T.L.; **FLECHER, M.C.**; CASSALI, G.D.; HORTA, R.S. Metronomic chemotherapy for advanced diffuse hepatocellular carcinoma in a dog. *BRAZILIAN JOURNAL OF VETERINARY PATHOLOGY*, v. 14, p. 199-205, 2021.

GORZA, L.L; OLIVEIRA, A. R.; PORTO, M. G; SANTOS, V. C.; HARDT, I.; MAESTRI, L.F. P.; SOUZA, T. D.; **FLECHER, M. C**. Clinical, anatomopathological, and immunohistochemical findings of a transitional cell carcinoma from nasal cavity, frontal and ethmoidal sinus with meningoencephalic invasion in a dog. *OPEN JOURNAL OF VETERINARY MEDICINE*, v. 11, p. 1-5, 2021.

MARQUES, C. A.; **FLECHER, M. C.**; FIDELIS JUNIOR, O. L.; BINDACO, A. L. S.; STEIN, J. D.; ROCHA, T. G.; OLIVEIRA, A. P. L. MESOTELIOMA PERITONEAL EPITELIOIDE PAPILAR MALIGNO EM BOVINO GIR - RELATO DE CASO. ARS VETERINÁRIA Jaboticabal, v. 37, p. 247, 2021.

3. Resumos em congresso relacionados a tese

FLECHER, M.; MONTEIRO, L.N.; SOUZA, R.R.; NAKAGAKI, K.Y.R.; CASSALI, G.D. CARACTERIZAÇÃO IMUNOFENOTÍPICA MOLECULAR DE NEOPLASIAS SÓLIDAS DA GLÂNDULA MAMÁRIA CANINA. I Congresso Latino de Patologia Mamária Canina e Felina & V Encontro de Patologia Mamaria. Belo Horizonte, 2023.

4. Resumos enviados para congresso/ eventos científicos não relacionados a tese

Flecher, M. C.; Santos, V. B.; Horta, R.S. COMPARAÇÃO DO INFILTRADO MASTOCITÁRIO EM LINFONODO DE CÃES E GATOS COM MASTOCITOMA, OUTRAS NEOPLASIAS E DOENÇAS NÃO-NEOPLÁSICAS - RESULTADOS PRELIMINARES. VII Encontro de Patologia da UFMG. I Simposio online de Patologia. 2020

FLECHER, M. C., LOPES, T.C.M., SOUZA, F.R., AVELAR, N.A., GEOVANNI DANTAS CASSALI, G.D. NEOPLASIAS PAPILARES DA GLÂNDULA MAMÁRIA DE CADELAS – CASUÍSTICA DE 15 ANOS DO LPC-UFMG. 6º Congresso Brasileiro de Patologia Veterinária e XX Encontro Nacional de Patologia Veterinária. Formato remoto. 2021

REYS, M.P., BINDACO, A.L.S., LUTZKE, D., MATIAS, C.H.T., SILVA, J.I., SOUZA, T.D., **FLECHER, M.C.** HEMANGIOSSARCOMA RENAL COM METÁSTASE CEREBRAL E PULMONAR EM CÃO: RELATO DE CASO. Congresso Brasileiro de Patologia Veterinária e XX Encontro Nacional de Patologia Veterinária. Formato remoto. 2021

FLECHER, M.C., OLIVEIRA, A. R., CARRETTA, M. SOUZA, F.R. CASSALI, G.D. ADENOMIOEPITELIOMA MALIGNO EM OURIÇO PIGMEU AFRICANO (ATELETRIX ALBIVENTRIS) DE ESTIMAÇÃO. XI ENDIVET. 2022

OLIVEIRA, M.; CARVALHO, T.S. GAVA, M.G., **FLECHER, M.C.** Correlação citológica e histopatológica de tumores da glândula mamária canina. I Congresso Latino de Patologia Mamária Canina e Felina & V Encontro de Patologia Mamaria. Belo Horizonte, 2023.

MATOS, L.S.F.; BINDACO, A.S.; REYS, M.P.; **FLECHER, M.C.** Carcinoma lobular pleomórfico mamário com invasão de cavidade torácica em cadela – relato de caso. I Congresso Latino de Patologia Mamária Canina e Felina & V Encontro de Patologia Mamaria. Belo Horizonte, 2023.

5. Capítulos de livro publicados.

CASSALI, G.D.; GRANDI, F.; **FLECHER, M.C.**; ABREU, C.C.; NAKAGAKI, K.Y.R.

Citopatologia Mamária: Técnicas de amostragem, interpretação e correlação cito-histológica. In: CASSALI, G. D.; NAKAGAKI, K.Y.R. Patologia Mamária Canina e Felina – Do diagnóstico ao tratamento. 2ed. São Paulo: Medvet, p.65-106, 2023.

NAKAGAKI, K.Y.R.; **FLECHER, M.C.**; CASSALI, G.D. Graduação histológica do câncer de mama. In: CASSALI, G. D.; NAKAGAKI, K.Y.R. Patologia Mamária Canina e Felina – Do diagnóstico ao tratamento. 2ed. São Paulo: Medvet, p.245-254, 2023.

DAMASCENO, K. A.; GARCIA, A.P.V.; SOUZA, F.R.; **FLECHER, M.C.**; CASSALI, G.D. Microambiente tumoral. In: CASSALI, G. D.; NAKAGAKI, K.Y.R. Patologia Mamária Canina e Felina – Do diagnóstico ao tratamento. 2ed. São Paulo: Medvet, p.455-478, 2023.

6. Participação em eventos científicos

Simpósio capixaba de Citopatologia Veterinária/ Jornada de Citopatologia da ABPV. 2020

Forum de ensino de Patologia Veterinária. 2020

6º Congresso Brasileiro de Patologia Veterinária e XX Encontro Nacional de Patologia Veterinária. Formato remoto. 2021.

I Congresso Latino de Patologia Mamária Canina e Felina & V Encontro de Patologia Mamaria. Belo Horizonte, 2023.

7. Organização de eventos científicos

Simpósio capixaba de Citopatologia Veterinária/ Jornada de Citopatologia da ABPV. 2020

Escola de Inverno da ABPV. 2020

Encontro de Patologia Veterinária - ENAPAVE 2020

Outubro Rosa Pet. 2021

I Congresso Latino-Americano de Patologia Mamária Canina e Felina e V Encontro de Patologia Mamaria. 2023

8. Palestras em eventos científicos

Mesa redonda: mulheres na ciência. Workshop de Carreira em Patologia Veterinária. 2021

Neoplasias da glândula mamária de machos. Outubro Rosa Pet 2021

OUTRAS REALIZAÇÕES DURANTE O DOUTORADO

1. **Revisora voluntária** para **The Brazilian Journal of Veterinary Medicine** (p-ISSN 0100-2430; e-ISSN 2527-2179)

2. Professora titular da disciplina de **PATOLOGIA VETERINÁRIA** da Universidade Vila Velha.
3. Orientadora de residência da área de **Patologia Animal** da Universidade Vila Velha.
4. Coordenadora de projetos de iniciação científica