



UNIVERSIDADE ESTADUAL DO CEARÁ
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS
DOUTORADO EM CIÊNCIAS VETERINÁRIAS

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**BIOMARCADORES IMUNOHISTOQUÍMICOS E SÉRICOS NA GLÂNDULA
MAMÁRIA CANINA: UMA ABORDAGEM DA FISIOLOGIA À NEOPLASIA**

FORTALEZA - CEARÁ

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Tese apresentada ao Curso de Doutorado em Ciências Veterinária do Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária da Universidade Estadual do Ceará em parceria com o Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto, como requisito parcial para obtenção do grau de Doutor em Ciências Veterinárias. Área de Concentração: Reprodução e Sanidade Animal.

Orientadores: Prof. Dr. Augusto Manuel Rodrigues Faustino e Profa. Dra. Lúcia Daniel Machado da Silva.

Coorientador: Prof. Dr. Geovanni Dantas Cassali.

FORTALEZA – CEARÁ

2025

Dados Internacionais de Catalogação na Publicação
Universidade Estadual do Ceará
Sistema de Bibliotecas
Gerada automaticamente pelo SidUECE, mediante os dados fornecidos pelo(a) autor(a)

Pinheiro, Breno Queiroz
Biomarcadores imunohistoquímicos e séricos na glândula
mamária canina: uma abordagem da fisiologia à neoplasia
[recurso eletrônico] / Breno Queiroz Pinheiro. - 2025.
99 f. : il.

Tese (DOUTORADO ACADÊMICO) -
Universidade Estadual do Ceará, Faculdade
de Veterinária, Curso de Programa de Pós-graduação Em
Ciências Veterinárias - Doutorado Acadêmico,
Fortaleza, 2025.

Orientação: Prof. Dr. Lucia Daniel Machado da Silva.

Coorientação: Prof. Dr. Geovanni Dantas Cassali.

1. ciclo estral. 2. CA 15-3. 3. Ki-67, marcadores
biológicos de tumor caspase-3. 4. prognóstico. 5. neoplasias
mamárias.. I. Título.

BRENO QUEIROZ PINHEIRO

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Universidade do Porto, como requisito parcial
para obtenção do grau de Doutor em Ciências
Veterinárias. Área de Concentração:
Reprodução e Sanidade Animal.

Tese aprovada em: 20/10/2025

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AGRADECIMENTOS

Este doutorado começou em um tempo de extremos. Nasceu em meio a uma pandemia global, um momento de incertezas coletivas, e em meio a um luto familiar, que marcou nossa história. A mesma família que chorou comigo foi também a que se fortaleceu ao meu lado. Reinventamo-nos juntos. Eles foram o pilar que sustentou os dias mais difíceis e celebraram as pequenas vitórias. A cada nova etapa, como em todas as anteriores, eles estiveram presentes. A eles, meu amor eterno e minha mais profunda gratidão.

Agradeço à minha mãe, Mayra Oliveira Queiroz, por sua presença constante, por cada gesto de cuidado, cada palavra de apoio e por nunca medir esforços para me ver bem. Mesmo sem compreender exatamente o que eu fazia em tantas fases deste percurso, ela sempre esteve ao meu lado em cada passo.

Agradeço também ao meu pai, Raison Barros Pinheiro. Ele foi o maior estímulo pessoal para que eu seguisse a Medicina Veterinária, e continua sendo inspiração diária.

Aos dois, meu mais profundo reconhecimento. Crescer os vendo como professores, indo à faculdade aos fins de semana, carregando provas me fez, desde cedo, admirar a sala de aula como um espaço de transformação. E foi exatamente essa convivência que me despertou o desejo de ser professor.

Agradeço ao meu companheiro, Pedro Arthur Figueiredo da Silva, por ser meu abrigo em meio ao caos. Em tantos momentos de tensão, prazos e exaustão, ele foi leveza, nos jantares improvisados de carbonara regados a vinho, nas conversas que me faziam relaxar e esquecer o relógio, nas viagens que me lembravam que existe vida para além dos artigos e planilhas.

Agradeço à Universidade Estadual do Ceará (UECE), minha casa desde os 17 anos. Nenhum outro lugar ocupou tanto tempo e espaço na minha trajetória quanto a UECE, não apenas como instituição, mas como local de descobertas, encontros, inquietações e amadurecimento. A UECE é, para mim, mais do que uma universidade: é um espaço simbólico que me formou e continua me formando.

A cada sala de aula, cada laboratório, cada biblioteca, cada pátio sombreado e cada cadeira desconfortável de sala de aula, meu muito obrigado. A todas as pessoas que cruzaram meu caminho nesses anos sejam professores, técnicos, colegas, servidores, terceirizados, vigilantes, motoristas, alunos, minha gratidão sincera. Nomear todos é tarefa impossível.

Agradeço à Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP), pela concessão da bolsa de estudos, e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pelo apoio por meio da taxa de bancada. Esse suporte foi fundamental para que as etapas deste doutorado fossem viáveis, garantindo as condições necessárias para a realização das atividades de pesquisa e para que este trabalho se concretizasse.

É impossível também e iniciar os agradecimentos dentro da UECE sem mencionar, antes de qualquer outro nome, a Professora Dra. Lúcia Daniel Machado da Silva, minha orientadora desde os meus 18 anos. A verdade é que, desde o início da graduação, eu já sabia: era no laboratório dela que eu queria estar, era com ela que eu queria aprender. E assim foi desde os primeiros passos na iniciação científica até aqui, neste doutorado que carrega tanto da sua escuta, da sua paciência, do seu rigor e do seu cuidado.

Não se faz pesquisa sem espaço. Não se faz pesquisa sem equipamento. E, mais do que isso, não se faz pesquisa sem pertencimento. Um dos espaços que foi o verdadeiro pontapé das minhas investigações acadêmicas e científicas foi o Laboratório de Reprodução de Carnívoros (LRC). A todos os membros desse laboratório, meu agradecimento mais sincero. Vocês foram, sem sombra de dúvida, os maiores formadores acadêmicos da minha trajetória profissional. Cada discussão, cada reunião de grupo, cada madrugada de pré-bancada deixou marcas que levarei por toda a vida.

Normalmente sou tomado por uma ansiedade que me impede de fazer uma coisa de cada vez. Durante a pós-graduação, vieram concursos, vieram aulas em instituições para além da UECE, vieram mil outras tarefas que abracei sem saber ao certo se daria conta. Mas, em meio a todas essas frentes, nenhuma marcou tanto minha trajetória quanto o Laboratório de Patologia Clínica Veterinária (LPCV) da UECE.

A esse espaço, deixo um agradecimento especial, não apenas pela estrutura, mas pelas pessoas. Nomear alguma delas é necessário, porque sem eles, nenhuma das minhas pesquisas teria saído do papel: Francisco Emanuel Pinheiro Cavalcante, Isabela Reis Nascimento, Marcely Braga de Albuquerque e Nathália Ferreira Carneiro. Obrigado é pouco. Vocês foram, e continuam sendo, fundamentais na minha caminhada.

Agradeço também ao professor Dr. Geovanni Dantas Cassali, responsável pelo Laboratório de Patologia Comparada da Universidade Federal de Minas Gerais (LPC/UFMG), a quem tive a honra de conhecer por intermédio da professora Lúcia. Ele foi peça absolutamente fundamental na construção deste doutorado, tenho plena convicção de que, sem o apoio, o conhecimento e a generosidade do professor Geovanni, eu sequer teria conseguido iniciá-lo.

Estendo meus agradecimentos à Dra. Fernanda Rezende Souza, que me recebeu com enorme gentileza na oportunidade em que estive presencialmente no LPC. A ela, sou grato não só pela acolhida, mas por toda a organização, logística e fluidez que proporcionou ao longo de cada etapa, especialmente diante das viagens intermináveis que as minhas amostras precisaram fazer para que este trabalho existisse.

Agradeço também à gentileza do professor Dr. Augusto Manuel Rodrigues Faustino, que, em plena pandemia mundial, aceitou receber um desconhecido vindo de outro país com uma disponibilidade e generosidade que me marcaram profundamente. Foi a receptividade do professor Augusto que me abriu o norte, permitindo que eu conhecesse Portugal e também horizontes para além das fronteiras da Medicina Veterinária.

Estendo meu agradecimento à professora Dra. Marta Susana Amaro dos Santos, que, ao lado do professor Augusto, me cedeu parte dos dados do seu doutorado, o que tornou possível a publicação do primeiro artigo científico do meu próprio doutorado.

Por fim, agradeço às Auroras, Cristais, Ninas, Lindinhas, Mels, Belinhas, Nanys, Aishas, Suris, e a todas as outras que cruzaram a minha trajetória ao longo deste doutorado. A elas, que nunca souberam o que era uma célula neoplásica, mas confiaram a mim seu corpo doente deixo minha mais profunda gratidão.

Agradeço também, e com igual importância, aos tutores que, em momentos de fragilidade, confiaram a mim a delicada responsabilidade de cuidar de quem tanto amam. Cada caso clínico foi mais do que um número, e parte essencial deste percurso acadêmico e humano.

“Sonho que se sonha só
É só um sonho que se sonha só
Mas sonho que se sonha junto é realidade.”

(Raul Seixas)

RESUMO

As neoplasias mamárias caninas representam uma das afecções tumorais mais prevalentes na clínica de pequenos animais e configuram um modelo consolidado para estudos comparativos em oncologia mamária. A identificação de biomarcadores prognósticos e a compreensão da dinâmica fisiológica da glândula mamária são essenciais para o aprimoramento diagnóstico e terapêutico. Esta tese teve como objetivo integrar dados da fisiologia mamária normal ao longo do ciclo estral com a avaliação de biomarcadores prognósticos séricos e teciduais em cadelas com neoplasias mamárias, visando propor parâmetros mais robustos para estratificação clínica. Foram conduzidos dois estudos complementares. O primeiro avaliou, por meio de dupla imunohistoquímica para Ki-67 e Caspase-3, a dinâmica proliferativa e apoptótica da mama canina normal em diferentes fases do ciclo estral, revelando aumento significativo do índice proliferativo no início do diestro, com forte correlação com os níveis séricos de progesterona, sobretudo em células epiteliais. O segundo estudo analisou o valor prognóstico do antígeno CA 15-3, dosado por imunoensaio enzimático com fluorescência, e das expressões teciduais de Ki-67 e COX-2 em 54 cadelas com neoplasias mamárias. Os resultados demonstraram que o CA 15-3 apresentou alta acurácia na diferenciação entre neoplasias malignas e benignas, além de correlação significativa com o estadiamento clínico e o grau histológico. O Ki-67 apresentou associação positiva com o grau histológico, reforçando seu papel como marcador de agressividade. Já a expressão de COX-2 mostrou-se inconsistente, com baixo valor prognóstico isolado, mas contribuiu para a caracterização multivariada dos tumores. A integração desses achados reforça a importância de considerar o contexto hormonal e fisiológico na interpretação de marcadores proliferativos, sugerindo que a associação entre biomarcadores séricos e teciduais pode aumentar a acurácia da estratificação prognóstica em neoplasias mamárias caninas. Esses resultados fortalecem o avanço de uma oncologia veterinária mais personalizada e apontam para o potencial translacional desse em modelo na compreensão comparativa do câncer de mama.

Palavras-chave: ciclo estral; CA 15-3; Ki-67, marcadores biológicos de tumor caspase-3; prognóstico; neoplasias mamárias.

ABSTRACT

Canine mammary neoplasms are among the most prevalent tumor conditions in small animal practice and constitute a well-established model for comparative breast oncology studies. The identification of prognostic biomarkers and a deeper understanding of the physiological dynamics of the mammary gland are essential for improving diagnostic accuracy and therapeutic strategies. This thesis aimed to integrate data from the normal physiology of the canine mammary gland across the estrous cycle with the evaluation of serum and tissue prognostic biomarkers in bitches with mammary neoplasms, in order to propose more robust parameters for clinical stratification. Two complementary studies were conducted. The first assessed the proliferative and apoptotic dynamics of the normal canine mammary gland through double immunohistochemistry for Ki-67 and Caspase-3 at different phases of the estrous cycle, revealing a significant increase in the proliferative index during early diestrus, strongly correlated with serum progesterone levels, especially in epithelial cells. The second study evaluated the prognostic value of the CA 15-3 antigen, measured by enzyme-linked fluorescent assay (ELFA), along with tissue expression of Ki-67 and COX-2 in 54 bitches with mammary neoplasms. The results demonstrated that CA 15-3 had high accuracy in distinguishing between malignant and benign tumors, with significant correlation to clinical staging and histological grade. Ki-67 was positively associated with histological grade, reinforcing its role as a marker of tumor aggressiveness. Conversely, COX-2 expression was inconsistent and showed limited prognostic value when analyzed independently but contributed to the multivariate characterization of tumor profiles. The integration of these findings underscores the relevance of hormonal and physiological context in interpreting proliferative markers and suggests that combining serum and tissue biomarkers enhances prognostic stratification in canine mammary tumors. These data support the advancement of a more individualized approach in veterinary oncology, with potential translational applications in comparative breast cancer models.

Keywords: Estrous Cycle; Mucin-1; Ki-67 antigen; Caspase-3; Prognosis.

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LISTA DE ABREVIATURAS E SIGLAS

115D8	Clone de anticorpo monoclonal dirigido ao antígeno MUC1/CA 15-3
A	Anestro (<i>anoestrus</i>)
AI	Índice apoptótico (<i>apoptotic index</i>)
ANOVA	Análise de variância (<i>analysis of variance</i>)
AUC	Área sob a curva ROC (<i>area under the curve</i>)
BC	Células basais totais (<i>total basal cells</i>)
CA 15-3	Antígeno do câncer 15-3 (epítipo de MUC1)
CEA	Antígeno carcinoembrionário (<i>carcinoembryonic antigen</i>)
CD31 ⁺	Células positivas para CD31/PECAM-1 (marcador endotelial)
CMN	Neoplasias mamárias caninas (<i>canine mammary neoplasms</i>)
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
COX-2	Ciclo-oxigenase 2
DF3	Clone de anticorpo monoclonal dirigido ao antígeno MUC1/CA 15-3
E	Estro (<i>oestrus</i>)
E ₂	17β-estradiol
EB	células de “end buds”/botões terminais (<i>end bud cells</i>)
EC	Células epiteliais totais (<i>total epithelial cells</i>)
ED	Diestro inicial (<i>early dioestrus</i>)
EDc	Células epiteliais de ductos (<i>epithelial cells of ducts</i>)
EIS	Epitélio das estruturas intralobulares (<i>epithelium of intralobular structures</i>)
FAVET/UECE	Faculdade de Veterinária da Universidade Estadual do Ceará
FI	Fator de impacto (<i>impact factor</i>)
FSH	Hormônio folículo-estimulante (<i>follicle-stimulating hormone</i>)
GMC	Glândula mamária canina
H&E	Hematoxilina e eosina
HBNs	Neoplasias mamárias humanas (<i>human breast neoplasms</i>)
HER-2	Receptor do fator de crescimento epidérmico humano 2
ICBAS/U.Porto	Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto
IFN-γ	Interferon gama
IgG1	Imunoglobulina G1
IgG2b-κ	Imunoglobulina G2b com cadeia leve kappa
IHC	Imuno-histoquímica (<i>immunohistochemical</i>)
IL-1β	Interleucina 1 beta
InSC	Células do estroma intralobular (<i>intralobular stroma cells</i>)
IntSC	Células do estroma interlobular (<i>interlobular stroma cells</i>)
iNOS ⁺	Células positivas para óxido nítrico sintase indutível (<i>inducible nitric oxide synthase</i>)
IU	Unidade internacional (<i>international unit</i>)
kD	Quilodalton (<i>kilodalton</i>)
Ki-67	Marcador de proliferação celular (proteína Ki-67)
LABPATVETUP	Laboratório de Patologia Veterinária da Universidade do Porto (<i>Veterinary Pathology Laboratory, UP</i>)
LD	Diestro tardio (<i>late dioestrus</i>)
LH	Hormônio luteinizante (<i>luteinizing hormone</i>)

M1–M5	Cadeias mamárias - torácicas cranial (M1) e caudal (M2), abdominais craniais (M3) e caudal (M4) e inguinal (M5)
MIB-1	Clone monoclonal MIB-1 anti-Ki-67
MKI67	Gene que codifica a proteína Ki-67
mL	Mililitro (<i>milliliter</i>)
MUC1	Mucina 1 (glicoproteína de membrana)
N	Componente “linfonodo” do estadiamento TNM
NMCs	Neoplasias mamárias caninas
NO	Óxido nítrico (<i>nitric oxide</i>)
P	Proestro (<i>proestrus</i>)
P ₄	Progesterona
PCA	Análise de componentes principais (<i>principal component analysis</i>)
PGE ₂	Prostaglandina E ₂
PI	Índice proliferativo (<i>proliferative index</i>)
Qualis	Sistema de classificação de periódicos da CAPES
RE	Receptores de estrogênio (<i>estrogen receptors</i>)
ROC	Curva característica de operação do receptor (<i>receiver operating characteristic</i>)
RP	Receptores de progesterona (<i>progesterone receptors</i>)
SD	Desvio padrão (<i>standard deviation</i>)
T	Componente “tumor” do estadiamento TNM
TGF-β	Fator de crescimento transformador beta (<i>transforming growth factor beta</i>)
Th1	Linfócitos T auxiliares tipo 1 (<i>T helper 1</i>)
TNF-α	Fator de necrose tumoral alfa (<i>tumor necrosis factor alpha</i>)
UFMG	Universidade Federal de Minas Gerais
VEGF	Fator de crescimento endotelial vascular (<i>vascular endothelial growth factor</i>)
τ (tau-b)	Coeficiente de correlação de Kendall tau-b

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1 INTRODUÇÃO

A glândula mamária canina é um órgão de alta complexidade morfofuncional, cuja atividade é profundamente modulada pelo ambiente hormonal do ciclo estral. A alternância entre as fases do ciclo reprodutivo influencia diretamente a taxa de renovação celular da glândula, tornando-a um modelo biológico relevante para o estudo de processos como proliferação e apoptose. Estudos demonstraram um aumento significativo da atividade proliferativa no início do diestro, correlacionado aos picos de progesterona, sobretudo em células epiteliais, evidenciando que a fisiologia hormonal exerce papel central na homeostase mamária (CHANDRA *et al.*, 2010; SANTOS *et al.*, 2010; PINHEIRO *et al.*, 2024).

Essa oscilação fisiológica dos índices celulares tem implicações diretas sobre a interpretação de biomarcadores amplamente utilizados em oncologia, como Ki-67 e Caspase-3 (PINHEIRO *et al.*, 2024). Por não serem exclusivos de processos neoplásicos, esses marcadores devem ser avaliados com cautela, especialmente em casos de lesões mamárias borderline, em que os níveis proliferativos podem refletir mais o momento fisiológico do ciclo do que uma real agressividade tumoral (ELCOMBE *et al.*, 2002; RODRIGUES *et al.*, 2016). Assim, compreender a fisiologia normal da mama canina ao longo do ciclo estral constitui um passo necessário para evitar erros diagnósticos.

No cenário das neoplasias mamárias caninas, que representam as neoplasias mais comuns em cadelas intactas, a estratificação prognóstica tem se beneficiado do uso de biomarcadores imunohistoquímicos e séricos (KASZAK *et al.*, 2022). O Ki-67 é amplamente empregado como marcador de proliferação celular e tem se mostrado útil na diferenciação de tumores de alto e baixo grau, enquanto a COX-2, associada a processos inflamatórios e angiogênese, apresenta expressão variável em tumores malignos, mas com menor poder prognóstico isolado (PEÑA *et al.*, 1998; PEREIRA *et al.*, 2009; ANADOL *et al.*, 2017; AYDOGAN *et al.*, 2018). Já o antígeno CA 15-3, tradicionalmente usado em oncologia humana, tem emergido como uma ferramenta promissora na veterinária, demonstrando boa acurácia na diferenciação entre tumores benignos e malignos, além de associação com estadiamento clínico e sobrevida (PINHEIRO *et al.*, 2023).

A análise combinada destes biomarcadores pode oferecer maior robustez na avaliação prognóstica, desde que interpretada à luz das particularidades fisiológicas da

mama canina (KASZAK et al., 2022; PINHEIRO et al., 2024). Diante da relevância do tema, a seguir apresenta-se uma revisão de literatura contemplando aspectos da glândula mamária canina e suas neoplasias, com ênfase em marcadores prognósticos.

2 REVISÃO DE LITERATURA

2.1 Glândula mamária canina (GMC)

A glândula mamária é uma estrutura exócrina especializada dos mamíferos, cuja função principal está relacionada à produção de leite para nutrição da prole. Nas fêmeas caninas, essas glândulas passam por intensas modificações morfológicas ao longo da vida, sobretudo em resposta às flutuações hormonais associadas ao ciclo estral, gestação e lactação. Já nos machos, as glândulas permanecem rudimentares, sem função secretora evidente. A presença de glândulas mamárias é um traço universal entre os mamíferos, considerado uma das principais características que os distinguem dos demais vertebrados (NOGUEIRA; CASTRO; GARCIA, 2023).

2.1.1 Aspectos morfofuncionais da GMC

A glândula mamária é uma glândula exócrina especializada derivada do ectoderma, formada a partir de espessamentos epiteliais bilaterais denominados cristas mamárias, que se estendem da região axilar à inguinal durante o desenvolvimento embrionário. Esses espessamentos originam brotos epiteliais que invadem o mesênquima subjacente, dando origem ao sistema ducto-alveolar. Embora compartilhe origem com as glândulas sudoríparas, a glândula mamária desenvolve características morfológicas e funcionais próprias. Embriologicamente, estabelece íntima relação com a pele, onde permanece inserida, e é funcionalmente subordinada ao controle do sistema endócrino, especialmente pelos hormônios ovarianos, hipofisários e placentários (NOGUEIRA; CASTRO; GARCIA, 2023; VALLE, 2023).

Anatomicamente, a glândula mamária da cadela é composta por múltiplas unidades lobulares organizadas em torno de sistemas ducto-alveolares (Figura 1). Cada lóbulo glandular é formado por numerosos ácinos e ductos, revestidos internamente por um epitélio simples cúbico a colunar, também denominado epitélio luminal ou

células alveolares, que repousa sobre uma camada descontínua de células mioepiteliais. Estas células mioepiteliais possuem natureza contrátil e estão interpostas entre o epitélio secretor e a membrana basal, envolvendo parcialmente as estruturas alveolares e ductais (NELSON, 1974; CHANDRA *et al.*, 2010; CASSALI *et al.*, 2023).

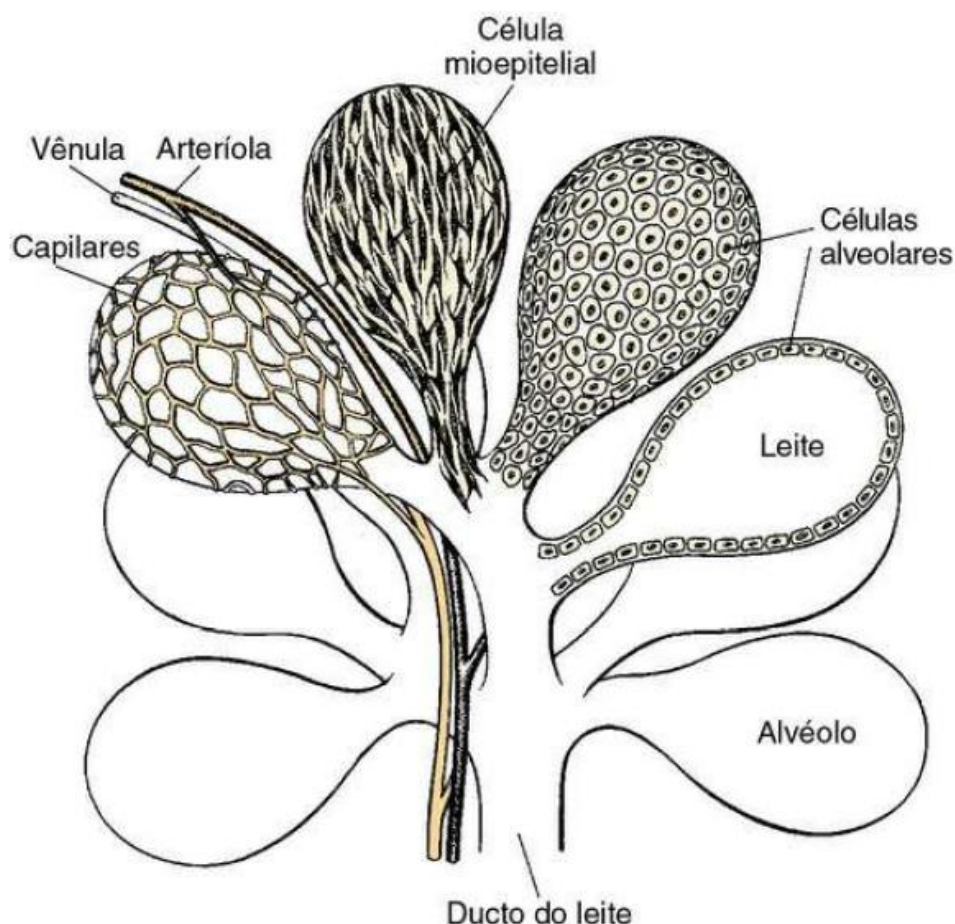


Figura 1 - Arquitetura Funcional do Lóbulo Mamário: Vascularização, Epitélio Secretor e Ductos Lactíferos.

Fonte: CUNNINGHAM, J. G. Tratado de Fisiologia Veterinária. 5. ed. Rio de Janeiro: Elsevier, 2021.

Os lóbulos glandulares são delimitados por septos de tecido conjuntivo interlobular, nos quais se distribuem vasos sanguíneos, linfáticos, fibras de colagénio, adipócitos e elementos imunológicos, como linfócitos e mastócitos. No interior de cada lóbulo, o tecido conjuntivo intralobular é mais frouxo e vascularizado, conferindo suporte e flexibilidade às estruturas glandulares. Os ductos lactíferos provenientes dos lóbulos convergem para ductos maiores, que por sua vez se dirigem ao seio lactífero, terminando

nos orifícios papilares localizados na região central de cada mamilo (SANTOS *et al.*, 2010; NOGUEIRA, CASTRO, GARCIA, 2023).

A disposição das mamas em cadelas segue um padrão bilateral simétrico (Figura 2), geralmente constituído por cinco pares, nomeados de cranial para caudal como torácicas cranial (M1) e caudal (M2), abdominais cranial (M3) e caudal (M4), e inguinais (M5), com a respectiva identificação do lado (direito ou esquerdo). Cada glândula mamária apresenta drenagem linfática preferencial: M1 e M2 direcionam-se principalmente aos linfonodos axilares superficiais; M4 e M5, aos linfonodos inguinais superficiais; já M3 possui drenagem variável, podendo alcançar tanto linfonodos axilares quanto inguinais. Essa organização, descrita por Cassali *et al.* (2023), Valle (2023) e Pimentel (2024), tem importância anatômica e clínica, especialmente para o planejamento diagnóstico e terapêutico de afecções mamárias.

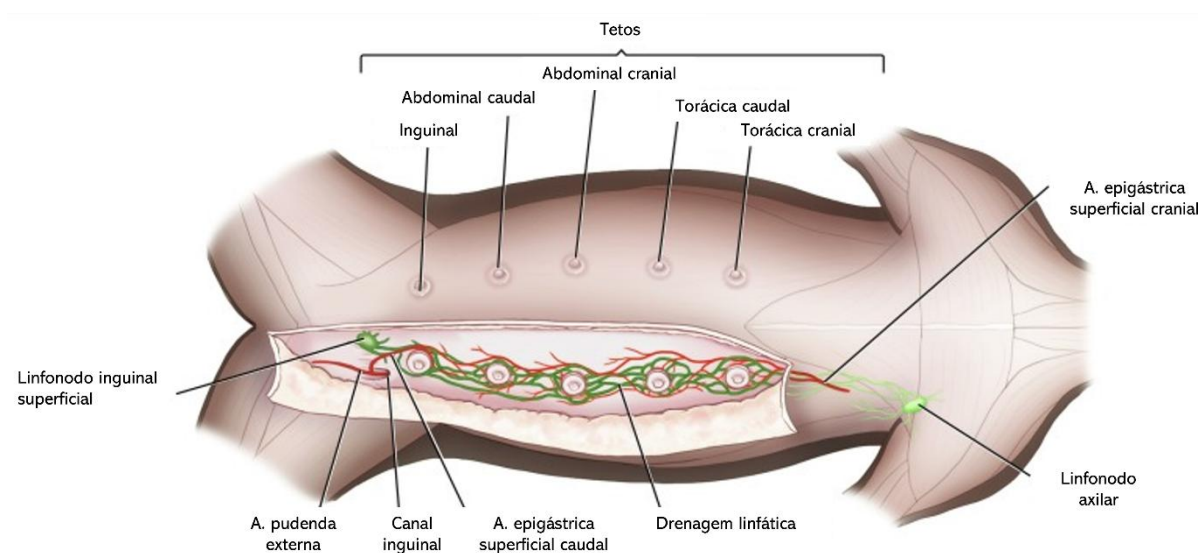


Figura 2 - Distribuição anatômica e drenagem linfática das glândulas mamárias em cadelas.

Fonte: Adaptado de KREKELER; DAVIES; BECK, 2022.

Do ponto de vista histológico (Figura 3), a estrutura glandular apresenta uma matriz extracelular rica em colágeno e fibras reticulares, que confere sustentação à arborização ductal e à manutenção da arquitetura lobular. As células epiteliais e mioepiteliais são sustentadas por uma membrana basal bem definida, cuja integridade é fundamental para a homeostase tecidual e delimitação das estruturas glandulares (KLÖPFLEISCH *et al.*, 2010; MICHEL *et al.*, 2012).

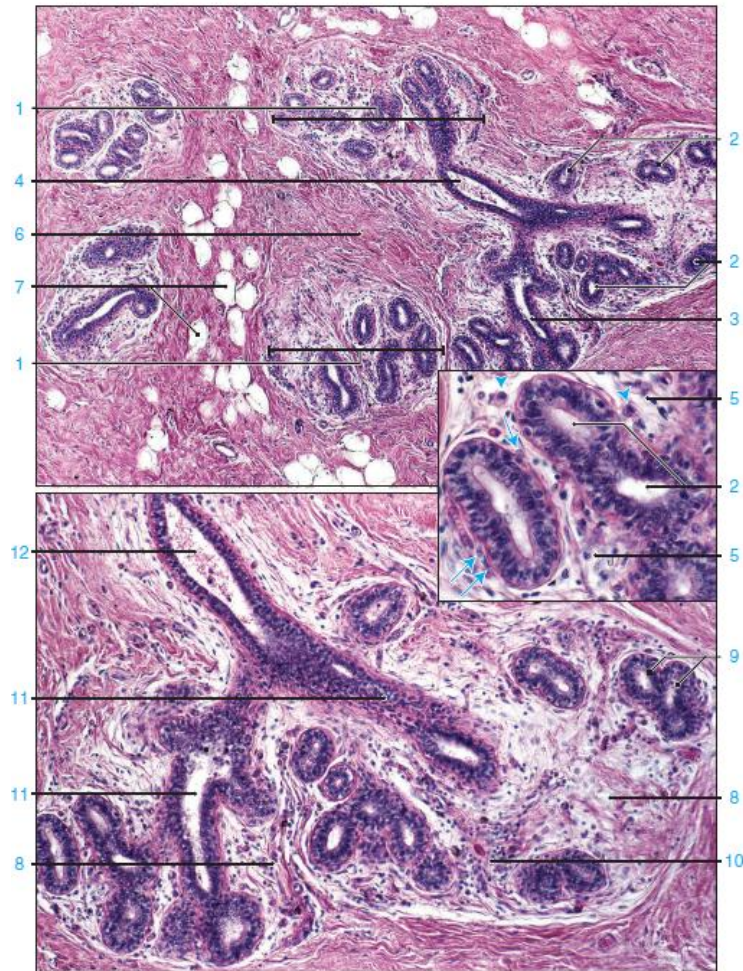


Figura 3 - Glândula mamária em repouso (H&E, 90×; detalhe ampliado: 350×). A imagem evidencia múltiplas unidades ducto-lobulares terminais (1), compostas por ductos terminais ramificados (2), cada um drenado por um ducto coletor intralobular (3) que, por sua vez, se conecta aos ductos interlobulares de maior calibre (4). As estruturas ductais encontram-se imersas em estroma intralobular frouxo e especializado (5), sensível à ação hormonal. Circundando os lóbulos, observa-se tecido conjuntivo interlobular denso (6), no qual estão dispersas células adiposas (7). A região ampliada na imagem inferior destaca novamente o estroma intralobular (8) envolvendo ductos terminais (9), com evidência de agregados linfocitários (10) e ductos intralobulares (11) conectando-se aos ductos interlobulares (12). Na ampliação, nota-se o epitélio cúbico dos ductos terminais e, em sua base, a presença de células mioepiteliais (setas azuis), além de plasmócitos e fibroblastos compondo o estroma especializado.

Fonte: Adaptado de ROSS, Atlas de histologia descritiva. Porto Alegre: Artmed, 2012.

2.1.2 Influência do ciclo estral na fisiologia da GMC

A GMC é altamente responsiva às variações hormonais que ocorrem ao longo do ciclo estral (Figura 4), que compreende as fases de proestro, estro, diestro e anestro. Cada uma dessas etapas é caracterizada por perfis hormonais distintos, que modulam diretamente a morfologia, a taxa de proliferação celular, os níveis de apoptose e a expressão de receptores hormonais. O estudo dessas alterações fisiológicas é fundamental para compreender não apenas o funcionamento normal do tecido mamário, mas também sua susceptibilidade a alterações displásicas e neoplásicas (SANTOS *et al.*, 2010; PINHEIRO *et al.*, 2024).

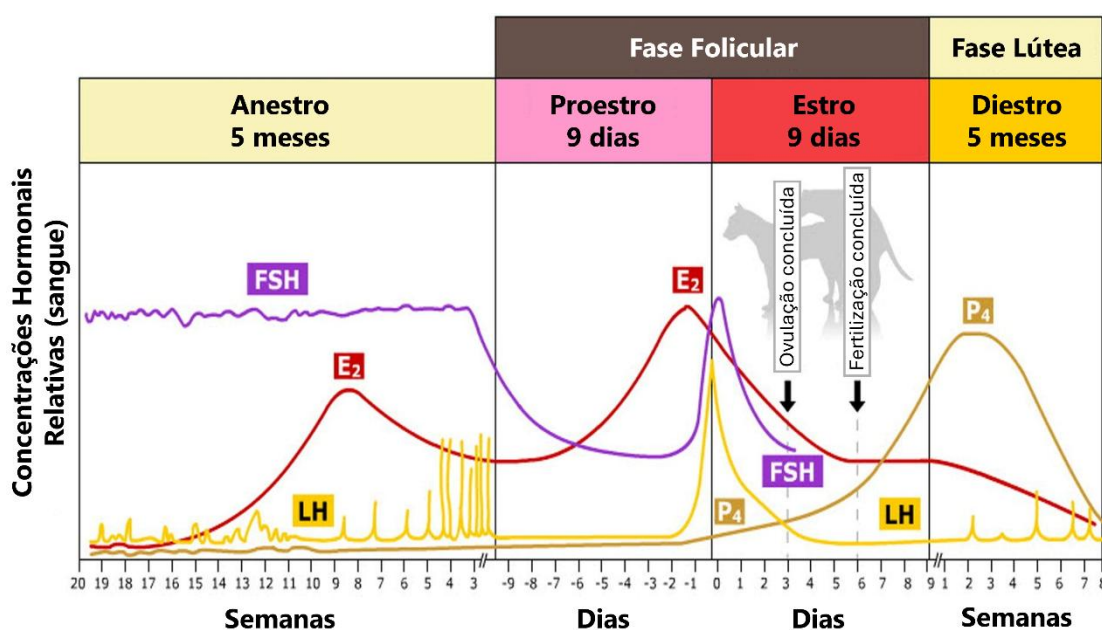


Figura 4 - Dinâmica hormonal durante o ciclo estral canino. Representação esquemática das concentrações relativas dos principais hormônios ovarianos (FSH, LH, estradiol – E₂ e progesterona – P₄) no sangue ao longo das fases do ciclo estral: anestro, proestro, estro e diestro. Observa-se que o pico de LH coincide com o final do proestro e é responsável por induzir a ovulação. O estradiol (E₂) atinge níveis elevados durante o proestro, enquanto a progesterona (P₄) se eleva no diestro, sustentando a fase lútea. As fases do ciclo estão delimitadas e organizadas em dias e semanas. As setas indicam o momento da ovulação e da fertilização, completando o ciclo reprodutivo típico da espécie.

Fonte: Adaptado de VETSCRAFT (2025).

No proestro, observa-se aumento progressivo das concentrações séricas de estrogênio, o que promove hipertrofia do epitélio ductal e aumento da vascularização

mamária (Figura 5). A atividade mitótica na glândula é moderada, e há aumento da expressão de receptores de estrogênio e prolactina, preparando o tecido para o estro subsequente. Histologicamente, os ductos tornam-se mais evidentes, com discreto edema intersticial e proliferação epitelial focal (REHM *et al.*, 2007; SANTOS *et al.*, 2010; MICHEL *et al.*, 2012).

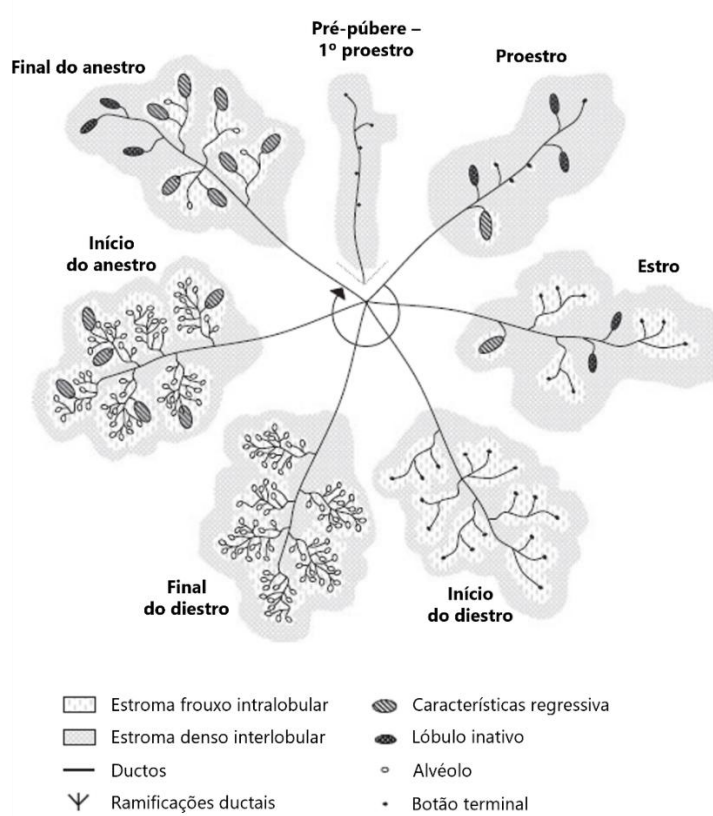


Figura 5 – Representação das características histológicas da glândula mamária durante o ciclo estral, incluindo a fase pré-púbere. Observa-se o desenvolvimento gradual das ramificações ductais, aparecimento de alvéolos e diferenciação lobular conforme a fase hormonal, com destaque para aumento do estroma frouxo intralobular e a presença de características regressivas nos períodos de final do diestro e anestro. A legenda indica os principais componentes histológicos envolvidos, como estroma interlobular denso, ductos, alvéolos, lóbulos inativos e estruturas regressivas.

Fonte: Adaptado de SANTOS *et al.*, 2010.

Durante a fase folicular, os níveis de estrogênio atingem o pico, enquanto a progesterona ainda permanece baixa. O epitélio mamário apresenta aumento da espessura e organização ductal mais definida. A proliferação celular continua moderada, e a expressão de receptores hormonais permanece elevada. Embora a diferenciação acinar

ainda seja discreta, o tecido demonstra atividade funcional crescente, refletindo prontidão para responder ao ambiente hormonal do diestro (DONNAY *et al.*, 1995; SPOERRI *et al.*, 2015).

O diestro é a fase de maior estímulo proliferativo, impulsionado pela elevação significativa dos níveis de progesterona, bem como pelo aumento da prolactina e do IGF-1. Nessa fase, há intenso desenvolvimento lobuloalveolar, mesmo na ausência de gestação, com diferenciação acinar proeminente e arquitetura glandular complexa. Marcadores como Ki-67 e PCNA apresentam expressão aumentada, e há forte ativação do eixo GH/IGF-1 autócrino. A apoptose celular é suprimida, e o tecido expande funcionalmente. Este ambiente pró-proliferativo é também o período de maior vulnerabilidade a alterações displásicas e neoplásicas (IMAOKA *et al.*, 2012; AYDOGAN *et al.*, 2018; STORNELLI *et al.*, 2020; PINHEIRO *et al.*, 2024).

Ao final do diestro, em cadelas não gestantes, observa-se involução lenta e incompleta da glândula, reflexo da ausência de luteólise abrupta. A apoptose epitelial começa a aumentar, mas de forma discreta e gradual. Esse padrão de involução, menos intenso do que o observado em modelos roedores, é marcado por declínio progressivo dos estímulos hormonais e retração funcional da glândula (HVID, 2012; STORNELLI *et al.*, 2020; PINHEIRO *et al.*, 2024).

Durante o anestro, os níveis hormonais estão reduzidos, especialmente estrogênio e progesterona, caracterizando um período de quiescência tecidual. A glândula apresenta arquitetura retraída, redução da proliferação celular, aumento da apoptose epitelial, e predominância do tecido conjuntivo interlobular (Figura 6) (CHANDRA *et al.*, 2010; SANTOS *et al.*, 2010).

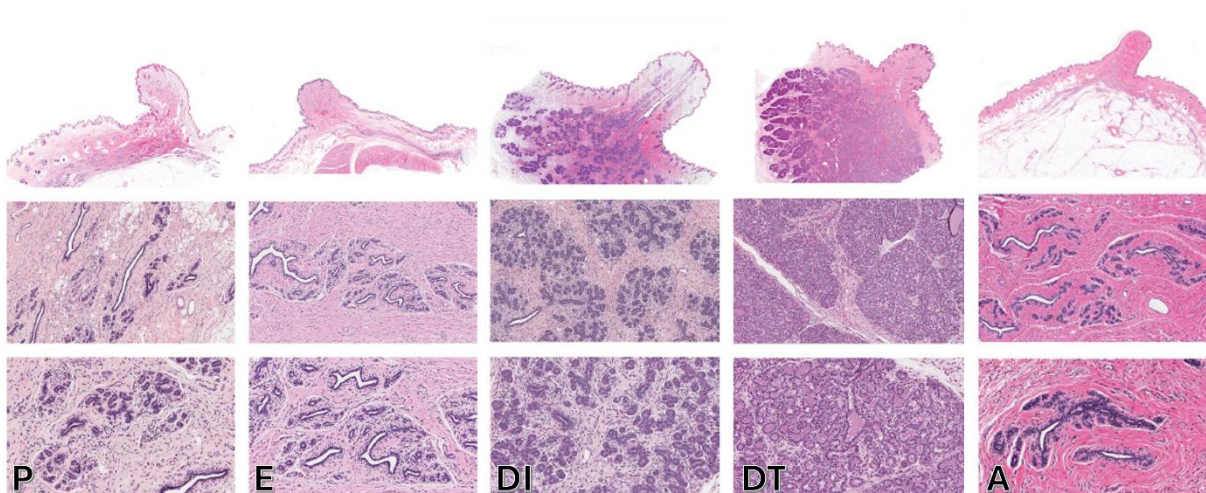


Figura 6 – Aspectos morfológicos da glândula mamária de cadelas Beagle ao longo do ciclo estral. As colunas mostram cortes histológicos representativos das fases do ciclo estral: proestro (P), estro (E), diestro inicial (DI), diestro tardio (DT) e anestro (A). Em cada fase, estão dispostas imagens em três níveis de aumento: submacroscópico (topo), aumento de 100x (meio) e aumento de 200x (base). Observa-se início da atividade glandular no proestro e estro, com diferenciação lobular. Há presença de secreção intraluminal no diestro inicial, há progressiva involução glandular no diestro tardio, e regressão acentuada no anestro, com predominância de ductos e estroma fibroso, aspecto semelhante ao observado no proestro. H&E.

Fonte: Adaptado de CHANDRA *et al.*, 2010.

Ao longo de todas as fases do ciclo, a expressão de receptores de estrogênio, progesterona e prolactina varia de forma coordenada com os níveis hormonais circulantes. Essas oscilações modulam diretamente o padrão funcional e morfológico da glândula, influenciando desde o crescimento epitelial até os mecanismos de morte celular programada. A persistência de estímulo hormonal, mesmo em ausência de prenhez, como ocorre na pseudogestação, pode favorecer o aparecimento de distúrbios proliferativos benignos e está implicada em eventos precoces da oncogênese mamária (MICHEL *et al.*, 2012; PINHEIRO *et al.*, 2024; REHM *et al.*, 2007).

2.2 Neoplasias mamárias caninas (NMCs)

Nos tópicos anteriores, demonstrou-se que a glândula mamária canina sofre modificações morfofuncionais significativas de acordo com a fase do ciclo estral e com os estímulos hormonais que a modulam. A transição de uma mama histologicamente normal

para uma glândula com lesões proliferativas benignas, e posteriormente para lesões malignas, é resultado da soma de múltiplas alterações genéticas e epigenéticas. De acordo com Polyak (2007), o desenvolvimento da neoplasia mamária segue uma sequência evolutiva iniciada, na maioria das vezes, em células epiteliais luminais que adquirem mutações somáticas, perdem a integridade da membrana basal e passam a se multiplicar descontroladamente, invadindo tecidos adjacentes e formando metástases (Figura 7).

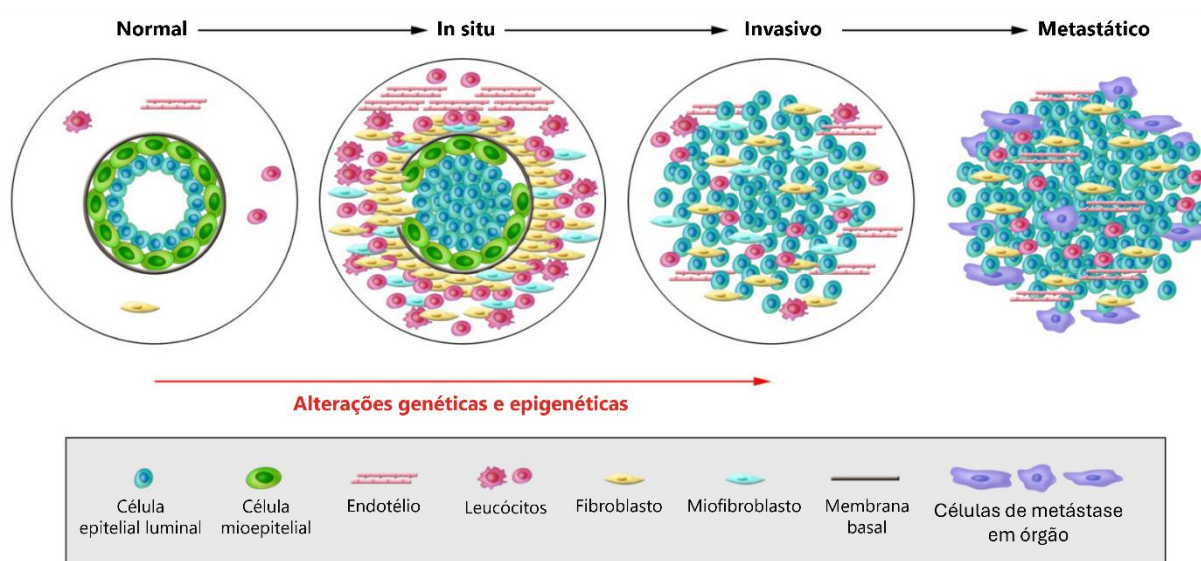


Figura 7 – Representação esquemática da progressão tumoral da glândula mamária: do tecido normal à metástase.

Fonte: Adaptado de POLYAK, 2007.

Hanahan e Weinberg (2000) já haviam sistematizado esse processo ao propor os “*hallmarks of cancer*”, segundo os quais a perda da homeostase celular envolve evasão de supressores tumorais, proliferação sustentada, angiogênese, invasão e metástase. Além disso, segundo Bissell e Radisky (2001), o estroma mamário não é apenas um coadjuvante, mas um elemento ativo na gênese tumoral, sendo capaz de induzir alterações malignas mesmo em células epiteliais inicialmente normais.

2.2.1 Aspectos epidemiológicos das NMCs

As NMCs são as neoplasias mais frequentes em cadelas, representando de 60% a 90% de todos os tumores nesse subgrupo da população. Trata-se de uma doença complexa e heterogênea, podendo levar a dificuldades em sua classificação,

diagnóstico e prognóstico (OLIVEIRA *et al.*, 2010; TORÍBIO *et al.*, 2012; NUNES *et al.*, 2018). Essa elevada prevalência, associada ao potencial invasivo e à ausência de políticas públicas efetivas de controle populacional em diversas regiões do mundo, confere às neoplasias mamárias importância crescente na medicina veterinária preventiva e oncológica (OLIVEIRA *et al.*, 2010).

A maioria das cadelas acometidas apresenta idade superior a oito anos, com média variando entre nove e onze anos. Fêmeas intactas estão sob maior risco, especialmente aquelas que passaram por múltiplos ciclos estrais. Raças de pequeno e médio porte, como Poodle, Cocker Spaniel e Dachshund, têm sido frequentemente citadas como predispostas, embora essa associação possa refletir também sua maior representatividade populacional. Quanto a apresentação clínica (Figura 8), as neoplasias mamárias caninas apresentam grande variabilidade clínica, podendo manifestar-se desde pequenos nódulos isolados até massas volumosas e ulceradas. Em casos avançados, podem atingir dimensões consideráveis, comprometendo a locomoção e o conforto do animal (GRÜNTZIG *et al.*, 2016; CASSALI *et al.*, 2020).



Figura 8 – Aspectos clínicos de neoplasias mamárias em cadelas. (A) Aumento difuso em placa da glândula mamária, compatível com carcinoma inflamatório, confirmado histopatologicamente. (B) Neoplasia de grandes proporções em glândula mamária caudal, histopatologicamente compatível com carcinoma em tumor misto.

Fonte: Próprio autor.

A influência hormonal é reconhecida como um dos principais fatores de risco na gênese das NMCs. Estudos clássicos sugerem que a castração precoce, antes do primeiro

ou segundo cio, reduz significativamente a probabilidade de desenvolvimento tumoral (SCHNEIDER *et al.*, 1969). Contudo, uma revisão sistemática conduzida por Beauvais *et al.* (2012) questionou a robustez desses dados, destacando a ausência de ensaios controlados e o risco de viés metodológico. A maior parte das evidências disponíveis é observacional e, portanto, limitada na sua capacidade de inferência causal (BEAUVAIS *et al.*, 2012).

Fatores como pseudogestação, gestação tardia, uso de contraceptivos hormonais e obesidade também têm sido associados ao aumento do risco de tumor mamário. A pseudogestação, em especial, estimula a proliferação mamária mesmo na ausência de gestação, e sua recorrência tem sido associada ao desenvolvimento de hiperplasias e displasias ductais, alterações que podem preceder o surgimento de neoplasias (CASSALI *et al.*, 2020; PINHEIRO *et al.*, 2024).

A realidade brasileira mostra proporção de malignidade consideravelmente maior que a reportada em estudos internacionais. Enquanto países de alta renda relatam entre 41–53% de tumores mamários caninos malignos (BRODEY *et al.*, 1983; VON EULER, 2011), no Brasil essa taxa varia de 68–90% (OLIVEIRA *et al.*, 2010; TORÍBIO *et al.*, 2012; NUNES *et al.*, 2018). Hipóteses para essa discrepância incluem: (I) diferença nos critérios histopatológicos/amostrais, incluindo a consideração de áreas *in situ*, microinvasivas e invasivas; (II) apresentação clínica tardia, com consequente progressão de lesões benignas para malignas; e (III) alta frequência de tumores mistos, com potencial de transformação maligna (FERREIRA *et al.*, 2009; CASSALI *et al.*, 2012; SORENMO; WORLEY; ZAPPULLI, 2020). Ademais, a baixa adesão à ovariectomia precoce, em contraste com países onde a castração antes dos primeirosaios é prática comum, eleva o risco populacional e pode contribuir para o cenário nacional (SCHNEIDER *et al.*, 1969).

A maior parte das neoplasias mamárias é de natureza epitelial. Os subtipos mais frequentes incluem os carcinomas complexos, carcinomas em tumor misto e carcinomas tubulares, seguidos pelos carcinomas sólidos e micropapilares (Figura 9). Tumores benignos, como o adenoma complexo e o tumor misto benigno, ainda são observados com frequência, especialmente em cadelas mais jovens ou castradas precocemente (MISDORP *et al.*, 1999; GOLDSCHMIDT *et al.*, 2011; CASSALI *et al.*, 2020; CASSALI *et al.*, 2024). A ocorrência de tumores múltiplos é comum, e a presença simultânea de tumores benignos e malignos em diferentes glândulas da mesma paciente ressalta a complexidade da doença mamária canina (SALAS *et al.*, 2015).

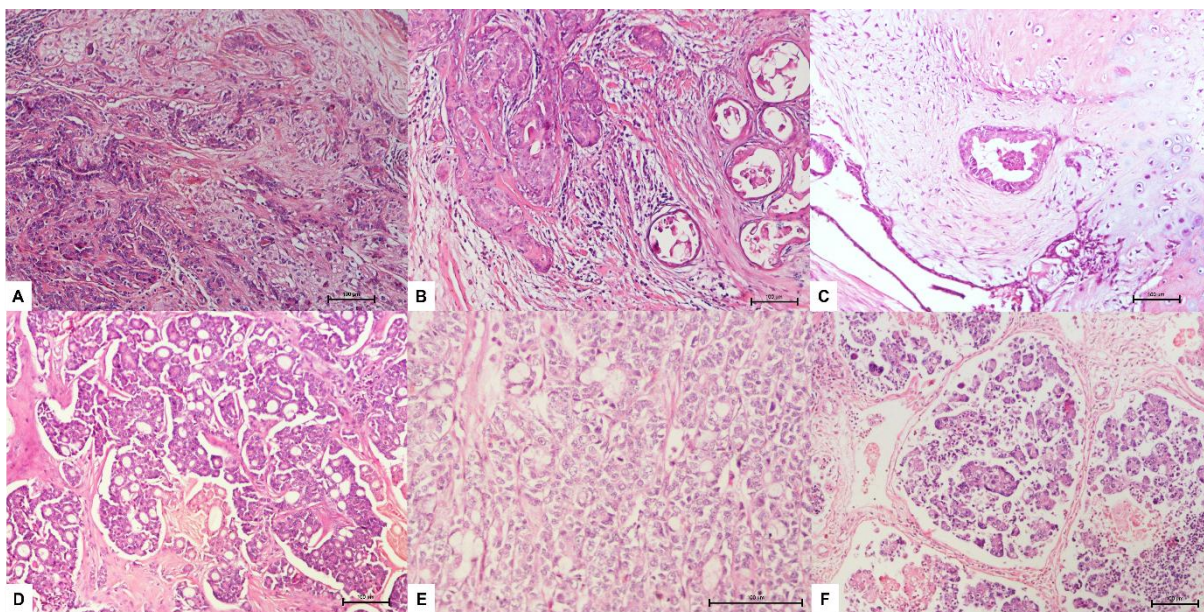


Figura 9 – Aspectos histopatológicos representativos de diferentes tipos de neoplasias mamárias em cadelas. A imagem apresenta cortes histopatológicos corados por H&E, obtidos com objetiva de 10× ou 20×, representando distintas classificações histopatológicas de neoplasias mamárias caninas: (A) **Adenoma complexo** – neoplasia benigna composta por proliferação de epitélio ductal e mioepitélio, em arquitetura organizada e com estroma fibroso discreto; (B) **Carcinoma complexo** – proliferação maligna com participação tanto epitelial quanto mioepitelial, com invasão focal do estroma e pleomorfismo moderado; (C) **Carcinoma em tumor misto** – componente epitelial maligno associado a matriz condroide, característica típica dos tumores mistos, com áreas de metaplasia; (D) **Carcinoma tubular** – proliferação glandular maligna com formação de túbulos bem organizados, associado a discreta resposta estromal; (E) **Carcinoma sólido** – proliferação celular densa em arranjos sólidos, com alto grau de celularidade e ausência de estruturas glandulares; (F) **Carcinoma micropapilar** – projeções papilares curtas e ausência de eixo fibrovascular. As barras de escala estão indicadas em cada painel.

Fonte: Próprio autor.

A diversidade de sistemas classificatórios utilizados ao longo das últimas décadas dificulta substancialmente a consolidação dos dados epidemiológicos. A classificação proposta por Misdorp *et al.* (1999), o sistema de Goldschmidt *et al.* (2011) e os consensos liderados por Cassali *et al.* a partir de 2011 apresentam diferenças nos critérios morfológicos, na terminologia e no enquadramento de subtipos histológicos. Essa heterogeneidade interfere diretamente na interpretação dos dados de incidência e

prevalência, além de dificultar comparações entre estudos e instituições. A adoção de critérios consensuais, como os estabelecidos nos documentos de Cassali *et al.* (2020; 2024), é fundamental para aprimorar a padronização diagnóstica e, por conseguinte, a precisão dos levantamentos epidemiológicos (MISDORP *et al.*, 1999; GOLDSCHMIDT *et al.*, 2011; CASSALI *et al.*, 2020; CASSALI *et al.*, 2024).

2.2.2 Fatores diagnósticos, prognósticos e preditivos

A avaliação diagnóstica das NMCs fundamenta-se primariamente no exame histopatológico, método considerado padrão-ouro. Ele permite a análise da arquitetura tumoral, das variações morfológicas celulares, da presença de mitoses e da graduação histopatológica. Essa graduação, baseada em critérios como pleomorfismo nuclear, índice mitótico e formação tubular, apresenta correlação direta com a agressividade biológica do tumor, sendo uma adaptação do sistema proposto por Elston e Ellis (1991), originalmente desenvolvido para carcinomas mamários humanos. Estudos como os de Rasotto *et al.* (2017) e Cañadas *et al.* (2019) demonstram uma correlação significativa entre o tipo histológico, o grau histopatológico e a sobrevida das pacientes.

Os fatores prognósticos referem-se a características clínicas, histopatológicas ou moleculares que, independentemente de tratamento adicional, permitem estimar a evolução clínica do paciente. Nas NMCs, são considerados fatores de prognóstico reservado o número e tamanho dos nódulos, presença de ulceração, grau histopatológico, metástase linfonodal e estadiamento clínico (FERREIRA *et al.*, 2009; ARAÚJO *et al.*, 2016; NUNES *et al.*, 2018; NUNES *et al.*, 2019). A heterogeneidade biológica observada entre os diferentes subtipos histológicos ainda representa um desafio para a estratificação prognóstica, como apontado por Cassali *et al.* (2020).

No contexto diagnóstico e prognóstico, a avaliação clínica pré-cirúrgica deve incluir anamnese reprodutiva detalhada, exame físico completo, hemograma, perfil bioquímico hepático e renal, além de exames de imagem, especialmente radiografia torácica em três projeções e ultrassonografia abdominal total. Esses exames são fundamentais para o estadiamento clínico e para a detecção de metástases ocultas (SORENMO; WORLEY; ZAPPULLI, 2020).

O estadiamento clínico das NMCs baseia-se no sistema T (tumor), N

(linfonodo) e M (metástase) (Quadro 1). Os estágios da doença avançam de I a III, conforme o tamanho tumoral aumenta de menos de 3 cm para mais de 5 cm (SORENMO; WORLEY; ZAPPULLI, 2020). A presença de metástases linfonodais caracteriza o estágio IV, e a ocorrência de metástases a distância define o estágio V. Esse sistema deve ser aplicado a neoplasias epiteliais, não inflamatórias e não mesenquimais. A determinação precisa do estadiamento clínico é essencial para definir a extensão da doença, estabelecer o prognóstico e planejar o tratamento mais adequado (CASSALI *et al.*, 2017; SORENMO; WORLEY; ZAPPULLI, 2020).

Quadro 1 – Estadiamento das neoplasias mamárias caninas.

Estágio	Tamanho do Tumor	Metástase linfonodal	Metástase à distância
I	< 3 cm	N ₀	M ₀
II	3 a 5 cm	N ₀	M ₀
III	> 5cm	N ₀	M ₀
IV	Qualquer T	N ₁ (positivo)	M ₀
V	Qualquer T	Qualquer N	M ₁ (metástase)

Fonte: Adaptado de SORENMO; WORLEY; ZAPPULLI, 2020.

Por outro lado, os fatores preditivos permitem estimar a resposta tumoral a tratamentos específicos, possibilitando a adoção de condutas terapêuticas mais individualizadas. Entre os biomarcadores com potencial preditivo mais avaliados destacam-se o Ki-67 (índice de proliferação), os receptores hormonais de estrogênio (RE) e progesterona (RP), a ciclooxigenase-2 (COX-2) e o antígeno tumoral CA 15-3 (KASZAK *et al.*, 2022).

A identificação e padronização desses marcadores vem ao encontro do avanço da oncologia de precisão, permitindo maior acurácia na avaliação de prognóstico e melhor seleção de terapias adjuvantes. Nesse cenário, a investigação contínua de fatores diagnósticos, prognósticos e preditivos nas NMCs torna-se fundamental para aprimorar a prática clínica e desenvolver estratégias personalizadas.

2.3 Biomarcadores moleculares aplicados à glândula mamária

Há uma crescente demanda por ferramentas diagnósticas que sejam sensíveis, específicas e minimamente invasivas, capazes de identificar precocemente alterações neoplásicas, mesmo antes do surgimento de sinais clínicos evidentes (SELTING *et al.*, 2013). Os biomarcadores neoplásicos são substâncias produzidas diretamente pelas células tumorais ou indiretamente pelo hospedeiro em resposta à presença do tumor, podendo ser detectadas em tecidos, sangue ou outros fluidos biológicos (CAMPOS, 2017; KASZAK *et al.*, 2018). Idealmente, essas moléculas deveriam ter concentração proporcional ao grau de malignidade, além de indicar o local do processo neoplásico; entretanto, nenhum marcador atual atende a esses critérios com sensibilidade e especificidade absolutas (CHEN *et al.*, 2020).

A medicina veterinária tem se beneficiado do conhecimento acumulado em oncologia humana, sobretudo no contexto das neoplasias mamárias, devido à notável semelhança molecular e biológica entre os tumores mamários de cadelas e mulheres (PEÑA *et al.*, 2014). Dentre os biomarcadores séricos mais investigados destacam-se o antígeno do câncer 15-3 (CA 15-3), o antígeno carcinoembrionário (CEA), sendo o CA 15-3 o mais promissor para diagnóstico precoce (CAMPOS, 2017).

Marcadores teciduais como Ki-67 e o COX-2 também têm sido amplamente empregues como ferramentas preditivas e prognósticas. A literatura aponta que a associação de múltiplos marcadores pode aumentar a acurácia diagnóstica, o que reforça o potencial desses biomarcadores na personalização do manejo clínico das NMCs (KASZAK *et al.*, 2022).

2.3.1 Ki-67 e proliferação celular

O Ki-67 é uma proteína nuclear codificada pelo gene MKI67, expressa exclusivamente em células ativamente proliferativas (G1, S, G2 e M), e ausente na fase G0. O anticorpo monoclonal MIB-1 é amplamente utilizado para a sua detecção devido à alta afinidade e confiabilidade na imunohistoquímica de tecidos fixados (Figura 10). Por essa razão, o Ki-67 é um dos marcadores mais utilizados em oncologia, auxiliando na avaliação do comportamento biológico dos tumores. Sua detecção permite quantificar a fração proliferativa - o chamado índice proliferativo – amplamente aplicado tanto na medicina humana quanto na veterinária para classificação e definição prognóstica das neoplasias (PEÑA *et al.*, 1998).

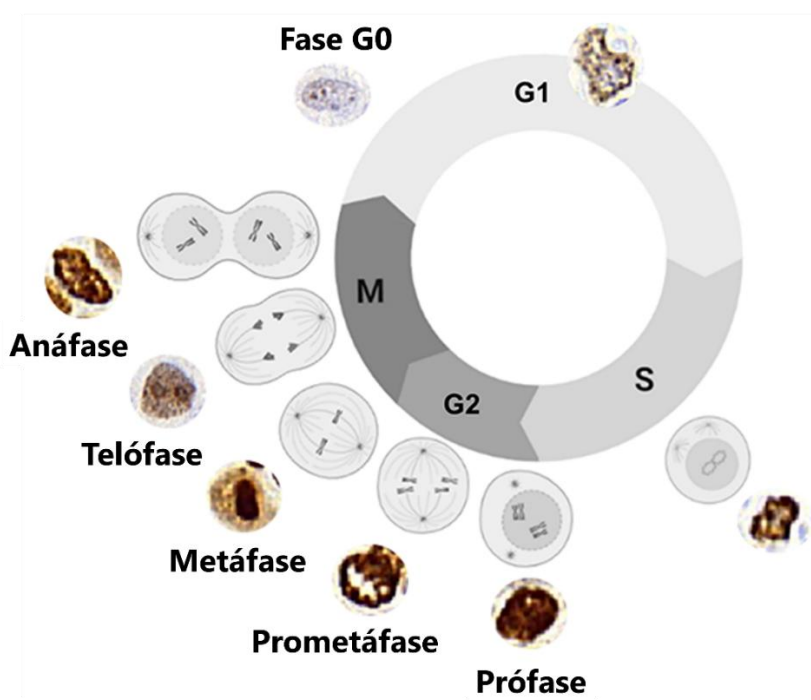


Figura 10 - Expressão do Ki-67 ao Longo das Fases do Ciclo Celular. O Ki-67 é detectado de forma dinâmica ao longo das fases do ciclo celular. Na fase G0, sua expressão é ausente. Durante a fase G1 inicial, surgem pequenos pontilhados nucleares que vão se agrupando, formando estruturas irregulares ao redor dos nucléolos na fase G1 tardia. Nas fases S e G2, o

Ki-67 se intensifica, distribuindo-se como grânulos densos assimétricos no nucleoplasma. Na prófase, sua localização se desloca para a camada pericromossomal. Já na prometáfase e metáfase, o Ki-67 se condensa junto aos cromossomos periféricos, enquanto os nucléolos não são mais identificáveis. Durante a anáfase e telófase, o gradiente de Ki-67 permanece elevado para garantir a separação cromossômica até a formação das células-filhas.

Fonte: Adaptado de FERNEZLIAN *et al.*, 2023.

Estudos demonstram que níveis elevados de Ki-67 estão associados à maior agressividade tumoral, estadiamento clínico mais avançado e grau histológico elevado. Isso significa que tumores com alto índice proliferativo tendem a apresentar maior capacidade de crescimento, invasão e metástase, influenciando negativamente a sobrevida dos pacientes. Tal correlação foi evidenciada por diversos autores, que relacionaram a expressão de Ki-67 ao aumento da taxa de recorrência e à redução da sobrevida livre de doença em cadelas acometidas por neoplasias mamárias (PEÑA *et al.*, 1998; CARVALHO *et al.*, 2016; RODRIGUES *et al.*, 2016; AYDOGAN *et al.*, 2018).

Além disso, há evidências de que o Ki-67 pode distinguir tumores benignos dos malignos. Em tumores benignos, sua expressão é significativamente menor, enquanto nos carcinomas mamários observa-se elevação expressiva, sobretudo nos subtipos mais agressivos, como carcinomas sólidos e micropapilares. Essa diferenciação auxilia não apenas na classificação histopatológica, mas também na definição de estratégias terapêuticas mais adequadas (AYDOGAN *et al.*, 2018).

Outro aspecto relevante é a relação inversa entre Ki-67 e outros marcadores biológicos, como caspase-3, marcador de apoptose. Essa correlação indica que tumores com alta taxa de proliferação celular tendem a apresentar menor índice apoptótico, contribuindo para o acúmulo de células neoplásicas e uma evolução clínica mais agressiva (RODRIGUES *et al.*, 2016).

Portanto, o Ki-67 é um biomarcador consolidado na oncologia veterinária, com ampla aplicabilidade no diagnóstico, prognóstico e monitoramento das neoplasias mamárias caninas. Sua associação com outros parâmetros clínico-patológicos fortalece a previsão do comportamento tumoral e promove uma abordagem mais precisa e personalizada na medicina veterinária.

2.3.2 Caspase-3 e apoptose

A apoptose é um processo de morte celular programada essencial à manutenção da

homeostase tecidual, promovendo a eliminação de células danificadas, potencialmente neoplásicas ou senescentes. Uma das principais proteínas envolvidas nesse processo é a caspase-3, considerada o “ponto final comum” tanto das vias extrínseca quanto da via intrínseca, promovendo a degradação de proteínas estruturais e nucleares que culminam com a morte celular ordenada (Figura 11). Em condições fisiológicas, esse processo impede a proliferação celular descontrolada e é regulado por uma complexa rede de proteínas pró e antiapoptóticas (PORTER; JÄNICKI, 1999).

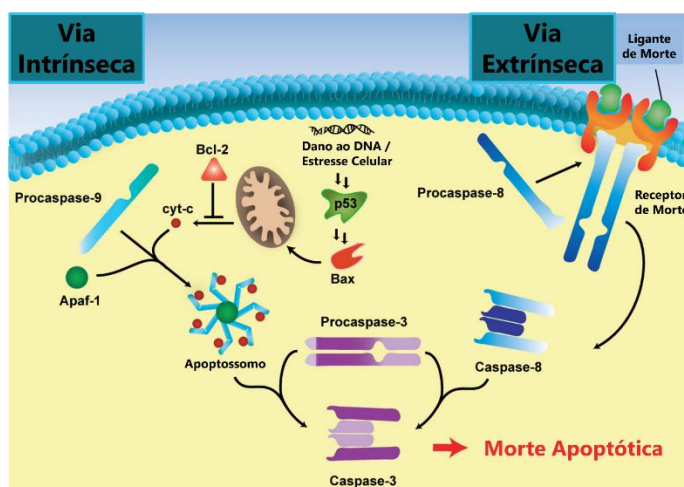


Figura 11 – Principais vias de ativação da apoptose celular mediadas por caspases. Representação esquemática das vias intrínseca e extrínseca de indução da apoptose. Na via intrínseca, o estresse celular ou dano ao DNA ativa a proteína p53, que estimula a liberação de citocromo c pelas mitocôndrias, levando à formação do apoptossomo e à ativação sequencial das caspases-9 e -3. Na via extrínseca, o reconhecimento de ligantes de morte por receptores específicos na membrana celular ativa a caspase-8, que também culmina na ativação da caspase-3. Ambas as vias convergem para a execução do processo de morte celular programada.

Fonte: Adaptado de HERGENROTHER LAB (2024).

No contexto das neoplasias mamárias caninas (NMCs), a expressão de caspase-3 tem sido extensivamente estudada como um marcador imunohistoquímico de apoptose. De forma geral, tumores malignos tendem a apresentar menor expressão de caspase-3 quando comparados aos benignos, evidenciando a capacidade das células tumorais em evadir os mecanismos de morte celular programada, favorecendo a progressão neoplásica (RODRIGUES *et al.*, 2016).

A associação entre a expressão de caspase-3 e o índice proliferativo Ki-67 tem sido alvo de diversas investigações. Estudos demonstram que quanto maior o índice proliferativo, menor

tende a ser a expressão de caspase-3, refletindo um desequilíbrio entre proliferação e apoptose nos tumores mais agressivos. Essa relação foi claramente demonstrada em carcinomas mamários de cadelas, nos quais o índice de apoptose diminui significativamente à medida que aumenta o grau histológico das neoplasias (AYDOGAN *et al.*, 2018).

Além dos dados obtidos na medicina veterinária, a literatura médica humana também sustenta a importância da caspase-3 como indicador prognóstico. Trabalhos como o de McConkey *et al.* (1996) demonstram que a ativação da caspase-3 está diretamente relacionada ao sucesso terapêutico e à sensibilidade tumoral à quimioterapia. A extrapolação desses achados para a oncologia veterinária sugere o potencial uso da caspase-3 como biomarcador preditivo em protocolos terapêuticos personalizados (MCCONKEY *et al.*, 1996).

Por fim, cabe destacar que o uso da caspase-3 na avaliação histopatológica das NMCs deve ser integrado a painéis multiparamétricos, associando-se a marcadores como Ki-67, Bcl-2 e receptores hormonais. A proposta de Kaszak *et al.* (2022) reforça a ideia de que marcadores isolados têm limitações diagnósticas e prognósticas, sendo mais efetivos quando utilizados em conjunto. Assim, a caspase-3 representa uma ferramenta valiosa não apenas para a compreensão da biologia tumoral, mas também para a construção de um modelo de avaliação mais acurado das neoplasias mamárias em cadelas (KASZAK *et al.*, 2022).

2.3.3 COX-2 e inflamação tumoral

A cicloxigenase-2 (COX-2) é uma enzima induzível envolvida na conversão do ácido araquidônico em prostaglandinas, mediadores lipídicos que desempenham papel central nos processos inflamatórios. Enquanto a COX-1 está associada a funções fisiológicas, como proteção gástrica e homeostase vascular, a COX-2 é expressa de forma transitória em tecidos inflamatórios e neoplásicos, sendo amplamente associada à carcinogênese em diversos tecidos, incluindo a glândula mamária canina (LAVALLE *et al.*, 2009).

A superexpressão de COX-2 em neoplasias mamárias caninas foi significativamente relacionada ao aumento da angiogênese, proliferação celular e infiltração inflamatória tumoral. O estudo de Carvalho *et al.* (2016) demonstrou que altos níveis dessa enzima estão fortemente associados a menor sobrevida, além de representar um fator de risco independente na análise multivariada de prognóstico tumoral. De forma complementar, Lavalle *et al.* (2009) identificaram correlação positiva entre COX-2 e densidade de microvasos CD31+, reforçando sua associação com estímulos angiogênicos.

A presença de inflamação no microambiente tumoral parece ser, inclusive, modulada pela atividade de COX-2. Pereira *et al.* (2009) relataram expressão aumentada da enzima em lesões malignas com infiltrado inflamatório acentuado, sugerindo que a resposta inflamatória tumoral pode ser, em parte, sustentada por prostaglandinas derivadas da via da COX-2. Da mesma forma, Anadol *et al.* (2017) observaram expressão de COX-2 em conjunto com iNOS e VEGF, o que aponta para um eixo inflamação-angiogênese, frequentemente presente em tumores de alto grau histológico (Figura 12).

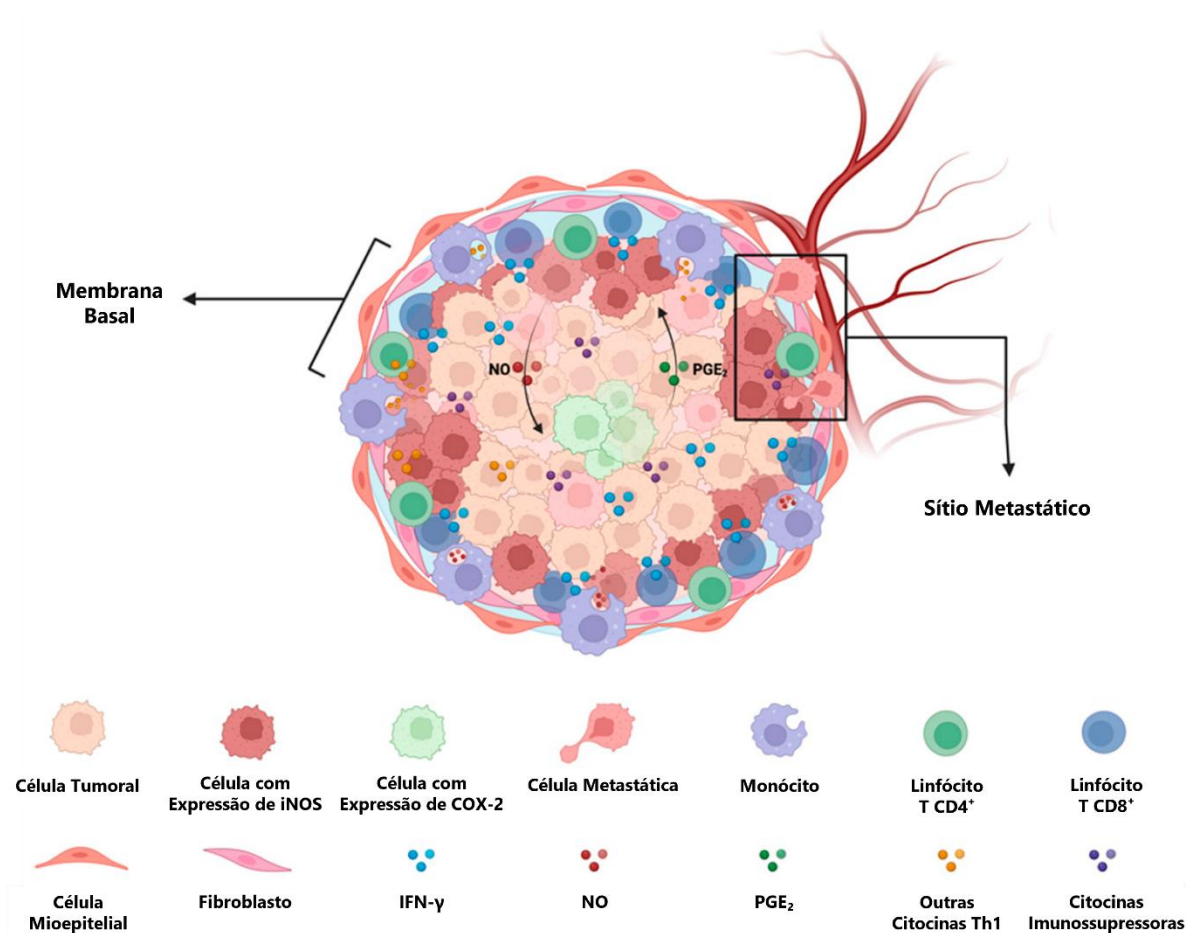


Figura 12 - Distribuição espacial e interação funcional entre células tumorais iNOS⁺ e COX-2⁺ no microambiente de carcinomas mamários. Representação esquemática do microambiente tumoral, destacando a expressão diferencial das enzimas iNOS e COX-2 em subpopulações de células tumorais. As células iNOS⁺ localizam-se predominantemente na periferia tumoral, próximas à membrana basal, em regiões ricas em células T CD8⁺, onde a produção local de óxido nítrico (NO) contribui para a exclusão imune e promoção de fenótipos metastáticos. Já as células COX-2⁺ concentram-se em regiões centrais do tumor, frequentemente pobres em infiltrado imune ("desertos imunológicos"), onde a produção de prostaglandina E₂ (PGE₂) reforça mecanismos imunossupressores. As vias iNOS-NO e COX-

2-PGE₂ interagem em um circuito de retroalimentação positiva, promovido por citocinas inflamatórias do tipo Th1 (como IFN- γ , TNF- α e IL-1 β), que culmina na indução de IL-10 e TGF- β , reforçando a imunotolerância tumoral. Esse arranjo funcional favorece a evasão imunitária, a progressão tumoral e a disseminação metastática.

Fonte: Adaptado de COUTINHO *et al.*, 2024.

Estudos adicionais mostram que a expressão de COX-2 pode estar presente desde glândulas não neoplásicas até carcinomas, porém com níveis crescentes à medida que aumenta o grau de malignidade. Esse achado foi reforçado por Queiroga *et al.* (2007), ao identificarem imunomarcção crescente de COX-2 conforme o grau histológico tumoral, inclusive em metástases linfonodais.

Por fim, deve-se destacar que a COX-2 também se associa a outras proteínas reguladoras da progressão tumoral. Araujo *et al.* (2016) demonstraram que sua expressão em metástases linfonodais está correlacionada com Ki-67 e HER-2, reforçando sua possível interação em eixos moleculares que promovem agressividade e resistência terapêutica. Assim, a COX-2 representa um elo entre inflamação, proliferação celular, progressão tumoral e potencial evasão imune, sendo uma molécula-alvo com potencial diagnóstico, prognóstico e terapêutico em tumores mamários de cadelas.

2.3.4 ARTIGO DE REVISÃO

The prognostic value of serum neoplastic biomarkers CA 15-3 and CEA in canine mammary neoplasms: a review

O valor prognóstico dos biomarcadores neoplásicos séricos CA 15-3 e CEA em neoplasias mamárias caninas: uma revisão

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Dados de publicação: *Rev Bras Reprod Anim*, v.46, n.3, p.290-297, jul./set. 2022. **Qualis:** C.
DOI: 10.21451/1809-3000.RBRA2022.021

Em seguida, apresenta-se nosso trabalho de revisão, publicado na Revista Brasileira de Reprodução Animal (Animal Reproduction), escolhida pelo alcance nacional em acesso aberto, pela comunicação direta com a rede de veterinários e pesquisadores brasileiros e por suprir uma lacuna de síntese crítica voltada à prática local, favorecendo a padronização de protocolos e a interpretação clínica em contexto brasileiro, com visibilidade também para a comunidade internacional. Essa publicação reuniu e analisou as principais evidências sobre a aplicabilidade desses marcadores em neoplasias mamárias caninas, discutindo desafios metodológicos e heterogeneidade de protocolos; sua inclusão nesta tese se justifica por oferecer base atualizada para a discussão dos achados específicos deste trabalho e por fortalecer o diálogo entre a realidade epidemiológica brasileira e as tendências recentes da literatura (PINHEIRO et al., 2022).

The prognostic value of serum neoplastic biomarkers CA 15-3 and CEA in canine mammary neoplasms: a review

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Resumo

As neoplasias mamárias caninas (NMCs) são as mais frequentes em cadelas, com isso, uma grande variedade de técnicas tem sido utilizada para identificar os seus fatores prognósticos. Dentre as possibilidades, os biomarcadores neoplásicos séricos antígeno do câncer (CA 15-3) e o antígeno carcinoembrionário (CEA) são os mais promissores. Biomarcadores neoplásicos são substâncias presentes na neoplasia, sangue ou demais produtos biológicos, produzidos pela neoplasia, ou secundariamente pelo paciente, em resposta à sua presença. Na medicina veterinária, esses marcadores são pouco estudados. Em termos gerais, eles não podem ser usados para o diagnóstico primário de neoplasias mamárias, mas estão relacionados com os fatores prognósticos já bem estabelecidos na literatura e tem como vantagem se tratar de uma análise menos invasiva e que é possível de ser feita de forma seriada. O objetivo desta revisão é descrever o CA 15-3 e o CEA como biomarcadores utilizados nas NMCs, o progresso recente feito na literatura e providenciar um sumário dos principais resultados já obtidos. O CA 15-3 e o CEA apresentam potencial prognóstico nas NMCs. Apesar de existir uma grande variação de resultados para esses biomarcadores na literatura, o seu uso deve ser considerado, visto os resultados obtidos na caracterização e prognóstico das NMC.

Palavras-chave: antígeno do câncer, antígeno carcinoembrionário, biomarcador tumoral, cão, tumor mamário.

Abstract

Female canine mammary neoplasms (CMNs) are the most frequent neoplasm in bitches, and a variety of techniques have been used to identify their prognostic factors. Among the possibilities, the serum biomarkers cancer antigen (CA 15-3) and carcinoembryonic antigen (CEA) are the most reliable. Neoplastic biomarkers are substances present in the neoplasia, blood, or other biological products, produced mainly by the neoplasia, or secondarily by the patient, in response to their presence. In veterinary medicine, these biomarkers are poorly studied. In general terms they cannot be used for primary diagnosis of mammary neoplasms but are related to the prognostic factors and have the advantage of being a less invasive analysis and that it is possible to be done in a serial way. The objective of this review is to describe CA 15-3 and CEA use as biomarkers in CMNs, the recent progress made in literature

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Recebido: 22/04/2021

Aceito: 21/07/2022



and the main overall results that had been already obtained. CA 15-3 and CEA have prognostic potential for CMNs. There is a wide variation of results for these biomarkers in literature, but despite this, its use must be considered as they provide relevant results in terms of characterization and prognostic in CMNs.

Keywords: biomarker tumor, cancer antigen, carcinoembryonic antigen, dog, mammary tumors.

Introduction

Female canine mammary neoplasms (CMNs) are the most common neoplasm in dogs, representing 50% to 70% off all neoplasms in this subset of population, and is a complex and heterogeneous disease with difficulties in its classification, diagnosis, and prognosis (Cassali *et al.*, 2020; Sorenmo, 2020). Studies suggest the annual incidence rate (IR) of CMNs was 111 to 250 cases per 100,000 dogs (Dobson *et al.*, 2002; Egenvall *et al.*, 2005; Vascellari *et al.*, 2020). These incidences are increasing, and annual incidence rate now are comparable to that in women (Vascellari *et al.*, 2020).

A variety of techniques have been used to identify new prognostic factors in CMNs (Webster *et al.*, 2011; Kaszak *et al.*, 2018; Cassali *et al.*, 2020). Among then, the serum biomarkers cancer antigen (CA 15-3), and carcinoembryonic antigen (CEA) are the most reliable (Mobasheri *et al.*, 2010).

Campos *et al.* (2012) performed Western blotting analysis to confirm the specificity and possible cross-reactivity of human CA 15-3 and CEA antibodies with canine serum and indicated a good interaction between human and canine CA 15-3 and CEA antibodies. Besides, Manuali *et al.* (2012) demonstrated a high similarity in the DF3 epitope of the Mucin 1 (MUC1), the target detected in the assays of CA 15-3, in human and dogs. This suggests that the determination of these biomarkers may be considered in bitches, using the same reagents kits used in humans.

Is known that is possible to use chemiluminescent (Marchesi *et al.*, 2007; Marchesi *et al.*, 2010; Manuali *et al.*, 2012; Campos *et al.*, 2012), radioimmunoassay (RIA) (Valencakova-Agyagosova *et al.*, 2012) and enzyme-linked immunosorbent assay (ELISA) technology (Campos *et al.*, 2015; Senhorello *et al.*, 2019; Fan *et al.*, 2021; Jain *et al.*, 2021; Ramadan *et al.*, 2021) for the determination of CA 15-3 and CEA in clinically healthy bitches and with CMNs (Table 1). The latter is the most common technique used for the detection in serum (Li *et al.*, 2019).

In cases of CMNs only have been published few papers evaluating the prognostic potential of CA 15-3 and CEA, with small and/or very heterogeneous groups of patients, using different molecular techniques, and a short follow up period (Marchesi *et al.*, 2007; Marchesi *et al.*, 2010; Manuali *et al.*, 2012; Campos *et al.*, 2012; Valencakova-Agyagosova *et al.*, 2012; Campos *et al.*, 2015; Roberto *et al.*, 2018; Baba *et al.*, 2019; Senhorello *et al.*, 2019; Fan *et al.*, 2021; Jain *et al.*, 2021; Ramadan *et al.*, 2021) (Table 1). None of these studies aimed to evaluate the effectiveness of these biomarkers in primary diagnosis.

In this light, the objective of this review is to summarize and described the prognostic value of serum neoplastic biomarkers CA 15-3 and CEA in CMNs, the recent progress made in literature with main overall results and some recommendations for clinical use of these biomarkers.

Cancer antigen 15-3 (CA 15-3)



After the hybridoma technology, developed by Köhler and Milstein (1975), becomes a routine tool, breast neoplasm cells were used to immunize BALB/c mice to generate monoclonal antibodies against neoplasm cell-associated antigens (Kufe *et al.*, 1984). Two such monoclonal antibodies, DF3 and 115D8, were used to develop an immunoassay, which is subsequently used to detect a specific cancer antigen named CA 15-3, CA15/3 or CA153 in the sera of breast neoplasm patients (Tondini *et al.*, 1988).

Further studies showed that the monoclonal antibody DF3 recognizes the core protein of Mucin 1 (MUC1) (Abe and Kufe, 1989) whereas the monoclonal antibody 115D8 recognizes part of the glycan chains on MUC1 (DAI *et al.*, 1998). The DF3 is an IgG1 whereas the 115D8 is an IgG2b-k with the apparent affinities of 5.26×10^{-9} and 1.84×10^{-9} M, respectively, to MUC1 (Dai *et al.*, 1998).

In humans and mice, MUC1 is normally expressed in the glandular or luminal epithelial cells of the mammary gland, esophagus, stomach, duodenum, pancreas, uterus, prostate, and lungs, and to a lesser extent, in hematopoietic cells (Gendler, 2001). In healthy tissues, MUC1 provides protection to the underlying epithelia creating a physical barrier limiting accessibility and preventing pathogenic colonization (Yolken and Peterson, 1992).

Most neoplastic cells present the transmembrane glycoprotein antigen MUC1 hypoglycosylated, redistributed over the cell surface and within the cytoplasm leading to a lack of cell polarity of the epithelial cells, these facts act as anti-adhesive mechanism and facilitating the detachment of malignant cells, and enabling MUC1 to be measured in serum (Nath and Mukherjee, 2014).

The importance of MUC1 in disease progression are related to neoplasm invasion, metastasis, expression of proangiogenic factors, production of growth factors, blocks hypoxia-induced cell death by mediating decreases in intracellular ROS, drug resistance by preventing the activation of the intrinsic apoptotic path-way and synthesis of IL-6 and TNF- α creating a proinflammatory milieu in the neoplasm microenvironment (Cheung *et al.*, 2000; Hollingsworth and Swanson, 2004; Cascio *et al.*, 2011; Nath and Mukherjee, 2014; David *et al.*, 2016).

CA 15-3 in canine mammary neoplasms

In CMNs cases is reported a significant increase in tissue membrane immunostaining of MUC1 compared to healthy ones (Manuali *et al.*, 2012). This allows MUC1 to be shed into the circulation where it can be measured by immunoassays as CA 15-3 (Hollingsworth and Swanson, 2004; David *et al.*, 2016).

Valencakova-Agyagosova (2012) proposes the cut-off value of 5.0 to 7.0 IU/mL in cases of CMNs, which are very similar that the cut-off reported by Jain *et al.* (2021) of 5.65 IU/mL. These results differ from the cut-off of 0,50 IU/mL proposed by Campos (2015), this difference might have occurred mainly because of different methodologies used, differences in clinical stage and histological grades of the patients in these works (Table 1). Until now, there are no reference intervals for CA 15-3 in the serum of normal bitches, a fact that makes further studies necessary.

Most studies show a statistical difference in serum CA 15-3 concentration between groups with and without CMNs (Marchesi *et al.*, 2010; Manuali *et al.*, 2012; Campos *et al.*, 2012; Valencakova-Agyagosova *et al.*, 2012; Campos *et al.*, 2015; Baba *et al.*, 2019; Fan *et al.*, 2021; Jain *et al.*, 2021; Ramadan *et al.*, 2021). Roberto *et al.* (2018), despite finding differences



in CA 15-3 serum dosages between canine females with and without CMNs, they demonstrated that the serum levels of CA 15-3 between patients with benign and malignant neoplasms were not significantly different, but most recently Fan *et al.* (2021) and Jain *et al.* (2021) also subdivide the mammary neoplasm group in benign and malign and reported a statistical difference between them.

When compared the serum concentrations with the clinical pathology parameters there is a significantly higher concentration of CA 15-3 in cases of bigger neoplasms (Campos *et al.*, 2012; Campos *et al.*, 2015; Jain *et al.*, 2021), lymph node metastasis (Campos *et al.*, 2012; Campos *et al.*, 2015; Fan *et al.*, 2021), higher histopathological grades (Manuali *et al.*, 2012; Fan *et al.*, 2021) and distant metastasis (Fan *et al.*, 2021) (Table 2). Valencakova-Agyagosova (2012) hypothesized that reproductive status can have possible effect on CA 15-3, but Roberto *et al.* (2018) observed that the animals' estrous cycle did not influence the values of the biomarker in canine females with CMNs.

The serum CA 15-3 concentration decreased significant post-surgery which may be good prognostic indicators and suggesting its possible use as a neoplasm recurrence control after mastectomy (Roberto *et al.*, 2018). Baba *et al.* (2019) despite not finding differences in the post-surgical period, found a lower mean survival in patients with higher serum concentrations of CA 15-3.

Carcinoembryonic antigen (CEA)

CEA is one of the best-known human neoplastic biomarkers discovered by Gold and Freedman in 1965 from colon neoplasm. They used an antibody obtained from a mouse previously immunized with an extract from hepatic metastases of an intestinal carcinoma. This antibody is produced by the induction of a surface glycoprotein involved in intracellular adhesion that contains 45 to 50% carbohydrates. The structure of this glycoprotein consists of a polypeptide chain consisting of 641 aminoacids, with lysine at its N-terminal position that has approximately 200 kD molecular weight (Grunnet and Sorensen, 2012).

CEA it is produced, mostly, by gastrointestinal mucosa, localized in epithelial cell membranes in small amounts, and it is overexpressed by epithelial cells of the colon, breast, and lungs (Kaszak *et al.*, 2018).

CEA has less sensitivity and specificity compared with CA 15-3 and for that is recommended as a biomarker of second option, subsidiary biomarker, which provides generally support to the main biomarker, the CA 15-3, and increased the detection of neoplasms (Valencakova-Agyagosova *et al.*, 2012).

CEA in canine mammary neoplasm

In dogs with mammary gland neoplasms, serum levels of CEA were elevated compared to healthy dogs (Valencakova-Agyagosova *et al.*, 2012; Baba *et al.*, 2019; Senhorello *et al.*, 2019; Jain *et al.*, 2021).

Table 1. Mainly results in literature for concentrations of CA 15-3 and CEA in serum of control bitches and with CMNs.

Authors	N	C	CMNs	Methodology	CEA (C) ng/mL	CA 15-3 (C) IU/mL	CEA (CMNs) ng/mL	CA 15-3 (CMNs) IU/mL
Marchesi <i>et al.</i> , 2007	105	30/31	44	Chemiluminescent	*	3 ± 0.14	*	2.73 ± 0.17**
Marchesi <i>et al.</i> , 2010	81	24	57	Chemiluminescent	*	0.57 ± 0.21	*	0.79 ± 0.56
Manuali <i>et al.</i> , 2012	50	*	50	Chemiluminescent	*	0.57 ± 0.21	*	0.80 ± 0.55
Campos <i>et al.</i> , 2012	90	30	60	Chemiluminescent	0.19 ± 0.20	1.19 ± 0.51	II (40) = 0.12 ± 0.12/ III (12) = 0.29 ± 0.36/ IV (8) = 0.07 ± 0.04	II (40) = 1.61 ± 0.61/ III (12) = 2.39 ± 1.02/ IV (8) = 2.46 ± 1.00
Valencakova-Agyagosova <i>et al.</i> , 2012	45	20	25	Radioimmunoassay	0.20 ± 0.03	5.14 ± 1.34	0.25 ± 0.06	8.58 ± 1.27
Campos <i>et al.</i> , 2015	48	20	28	ELISA	*	0.31 ± 0.19	*	II (14) = 0.53 ± 0.45/ III (14) = 1.08 ± 0.44
Roberto <i>et al.</i> , 2018	40	40	40	Chemiluminescent	*	0.19 ± 0.39	*	1.56 ± 0.39
Baba <i>et al.</i> , 2019	47	6	41	ELISA	1.03 ± 0.17	1.25 ± 0.04	1.84 ± 0.09	1.33 ± 0.01
Senhorello <i>et al.</i> , 2019	77	21	56	ELISA	0.60 ± 0.34	*	II (31) = 1.50 ± 0.71/ III (12) = 2.19 ± 0.77/ IV (13) = 2.76 ± 0.73	*
Fan <i>et al.</i> , 2021	178	40	138	ELISA	*	*	*	*
Jain <i>et al.</i> , 2021	60	20	40	ELISA	201.03 ± 48.54	B = 281.08 ± 83.75/ M = 377.92 ± 65.80c	5,02 ± 0,90	B = 5,91 ± 0,60/ M = 7,71 ± 0,88
Ramadan <i>et al.</i> , 2021	17	7	10	ELISA	0.14 ± 0.03	0.15 ± 0.05	1,33 ± 0,10	3,76 ± 0,43

N = Number of animals. C = Number of animals in the control group. * Not reported. ** Not only dogs with CMNs were evaluated. Campos *et al.*, 2012: II (T1,2,3N0M0); III (T1,2,3N1,2M0); IV (T1,2,3N1,2M1). Campos *et al.*, 2015: II (T1,2,3N0M0); III (T1,2,3N1,2M0). Senhorello *et al.*, 2019: II (T1N0M0); III (T2,3N0M0); IV (T1,2,3N1,2M0). Jain *et al.*, 2021: B = Benign mammary neoplasms; M = Malignant mammary neoplasms.



Table 2. Main related results in literature of CA 15-3 and CEA with the prognostic factors in CMNs.

Authors	Biomarker	Statistical difference	Cut-off	Size related	Grade related	Lymph node metastasis related	Decreasing post-surgery
Marchesi <i>et al.</i> , 2007	CEA	*	**	**	**	**	**
	CA 15-3	No	**	**	**	**	**
Marchesi <i>et al.</i> , 2010	CA 15-3	Yes	**	**	**	**	**
Manuali <i>et al.</i> , 2012	CA 15-3	Yes	**	No	Yes	**	**
Campos <i>et al.</i> , 2012	CEA	No	**	**	**	**	**
	CA 15-3	Yes	**	Yes	**	Yes	**
Valencakova-Agyagosova <i>et al.</i> , 2012	CEA	Yes	0.20–0.23 ng/mL	**	**	**	**
	CA 15-3	Yes	5.0–7.0 IU/mL	**	**	**	**
Campos <i>et al.</i> , 2015	CA 15-3	Yes	0.50 IU/mL	Yes	**	Yes	**
Roberto <i>et al.</i> , 2018	CA 15-3	Yes	**	**	**	**	Yes
Baba <i>et al.</i> , 2019	CEA	Yes	**	**	**	**	Yes
	CA 15-3	Yes	**	**	**	**	Yes
Senhorello <i>et al.</i> , 2019	CEA	Yes	1.08 ng/mL	Yes	Yes	Yes	Yes
Fan <i>et al.</i> , 2021	CEA	No	**	**	Yes	Yes	**
	CA 15-3	Yes	**	**	Yes	Yes	**
Jain <i>et al.</i> , 2021	CEA	Yes	247.65 ng/L	**	**	**	**
	CA 15-3	Yes	5.65 IU/mL	Yes	**	**	**
Ramadan <i>et al.</i> , 2021	CEA	No	**	**	**	**	**
	CA 15-3	Yes	**	**	**	**	**

* Not able to be measured. ** Unreported.

CEA serum values in canine females without CMNs is approximately ten times smaller than in humans and ranged from 0.00 to 0.23 ng/mL (Campos *et al.*, 2012).

As observed for CA 15-3, there is a wide variation of results for CEA, and once more this difference mainly occurred probably because of different methodologies used and the use of different reagents (Table 1) (Campos *et al.*, 2012; Valencakova-Agyagosova *et al.*, 2012; Baba *et al.*, 2019; Senhorello *et al.*, 2019; Fan *et al.* 2021; Jain *et al.*, 2021; Ramadan *et al.*, 2021).

CEA is a neither specific nor sensitive biomarker for primary diagnosis of CMNs but can be a subsidiary biomarker to detect CMNs (Jain *et al.*, 2021). CEA level is aimed for assigning a prognosis and follow-up, to detect recurrence in patients who have undergone surgery, and to monitor the therapeutic response (Valencakova-Agyagosova *et al.*, 2012; Baba *et al.*, 2019; Senhorello *et al.*, 2019) (Table 2).

Senhorello *et al.* (2019) propose the CEA cut-off value of 1.08 ng/mL for malignant CMNs detection, which is very discrepant of the result found by Jain *et al.* (2021) of 247.65 ng/L. This difference can be explained by the same justification used in CA 15-3.

The serum CEA concentration was found to be lowest in healthy bitches, higher in bitches with benign neoplasms, and highest in bitches with malignant CMNs (Jain *et al.*, 2021). The concentration of CEA was also higher in bigger neoplasms (Senhorello *et al.*, 2019), lymph node metastasis (Senhorello *et al.*, 2019; Fan *et al.* 2021), higher histopathological grades (Senhorello *et al.*, 2019; Fan *et al.* 2021) and distant metastasis (Fan *et al.*, 2021) (Table 2).

CEA values started decreasing 15 days after the neoplasm was removed, which may be a good prognostic indicator, but no significant difference was observed when compared with the presurgical levels (Baba *et al.*, 2019; Senhorello *et al.*, 2019), suggesting that further studies aiming at greater follow-up should be performed.

Recommendations for clinical use in CMNs

Since these biomarkers are relatively easy, less invasive, and cheap to determine compared with immunohistochemical tests, preoperative levels might be combined with existing prognostic factors in planning the optimum CMNs management. Even after mastectomy we recommend serial levels measurements of these serum biomarkers during the follow-up, particularly of asymptomatic patients, as increases in the concentration should be related with a worst outcome, and in this case additional procedures must be performed. Also, the sensitivity and accuracy of the combined detection of these neoplastic biomarkers are significantly higher than that of single detection.

Concluding remarks

Human kits for CA 15-3 and CEA have proven to be useful in bitches with CMNs. CA 15-3 and CEA cannot be used for screening the general asymptomatic population or for independently diagnosing in cases of CMNs, therefore they have been proven to be correlated to the prognostic factors and could play an important role in the management and follow-up of patients. Most of the studies in literature regarding CA 15-3 and CEA in CMNs evaluate only a small group of patients and/or very heterogeneous groups and use different molecular techniques. Looking into the future, more detailed studies should be carried out with a large prospective randomized trial and with the emphasis moving from single to multiple biomarkers, especially in screening for early disease, and in focusing on a longer follow-up evaluation.

Acknowledgements

The authors thank the Coordination for the Improvement of Higher Education Personnel (CAPES) for granting a scholarship during this study. The authors are also grateful to the National



Council for Scientific and Technological Development (CNPq; Project numbers 305420/2013-5) for research Support.

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3 JUSTIFICATIVA

As neoplasias mamárias caninas representam um dos principais desafios da oncologia veterinária, com elevada prevalência e significativa heterogeneidade biológica, histológica e clínica. A crescente busca por abordagens individualizadas de prognóstico e terapia demanda a identificação de biomarcadores confiáveis, não apenas em tecidos neoplásicos, mas também em fluidos biológicos, como o sangue.

Apesar do avanço na caracterização histopatológica dos tumores mamários, ainda há lacunas quanto à integração entre biomarcadores séricos, perfis imuno-histoquímicos e os processos fisiológicos da mama canina, especialmente em relação às variações hormonais do ciclo estral, que podem influenciar a interpretação dos dados proliferativos e apoptóticos. Neste contexto, os estudos desenvolvidos nesta tese visam preencher essas lacunas: avaliação do comportamento dinâmico dos índices proliferativos e apoptóticos na mama normal ao longo do ciclo estral; investigar o valor prognóstico de três importantes biomarcadores (CA 15-3, Ki-67 e COX-2) em tumores mamários caninos, com ênfase na estratificação clínica e análise de sobrevida.

4 HIPÓTESE CIENTÍFICA

Os níveis de expressão de biomarcadores como CA 15-3, Ki-67, COX-2 e Caspase-3 variam de forma significativa de acordo com a agressividade clínica e histopatológica das neoplasias mamárias caninas, e sua interpretação pode ser refinada à luz do conhecimento sobre a fisiologia mamária normal ao longo do ciclo estral.

5 OBJETIVOS

5.1 Objetivo geral

Investigar os aspectos morfofuncionais da glândula mamária canina normal durante o ciclo estral e avaliar o valor prognóstico de biomarcadores séricos e teciduais (CA 15-3, Ki-67 e COX-2) em cadelas com neoplasias mamárias, visando propor um modelo integrativo de estratificação diagnóstica e prognóstica.

5.2 Objetivos Específicos

- a) Determinar os índices proliferativo (Ki-67) e apoptótico (Caspase-3) em diferentes compartimentos histológicos da glândula mamária canina ao longo das fases do ciclo estral.
- b) Avaliar a correlação entre os níveis hormonais (progesterona e estradiol) e os índices proliferativo e apoptótico da glândula mamária normal.
- c) Medir os níveis séricos de CA 15-3 em cadelas com diferentes estágios de neoplasia mamária e compará-los com cadelas saudáveis.
- d) Avaliar a expressão tecidual de Ki-67 e COX-2 em amostras de neoplasias mamárias caninas, correlacionando com grau histológico, estadiamento clínico e sobrevida.
- e) Investigar o valor preditivo e discriminativo de cada biomarcador (isoladamente e em combinação) na diferenciação entre neoplasias benignas e malignas.
- f) Propor uma abordagem integrada para interpretação dos biomarcadores, considerando tanto os dados fisiológicos da glândula normal quanto os parâmetros clínico-patológicos dos tumores.

6 CAPÍTULO I

Proliferation and apoptosis dynamics of the normal canine mammary gland during the oestrous cycle evaluated by Ki-67 and Caspase-3 indexes

Dinâmica de proliferação e apoptose da glândula mamária canina normal ao longo do ciclo estral avaliada pelos índices Ki-67 e Caspase-3

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Dados de publicação: *Reprod. Domest. Anim.*, 2024, 59(6):e14655. Recebido: 31 jan. 2024.

Aceito: 11 jun. 2024. **FI:** 1,76. **Qualis:** A3.

DOI: 10.1111/rda.14655.

Em seguida apresenta-se o artigo publicado que fundamenta o Capítulo 1. O estudo estabelece parâmetros de referência para a fisiologia mamária canina ao longo do ciclo estral, quantificando sistematicamente os índices de proliferação (Ki-67) e apoptose (Caspase-3) em diferentes estruturas da cadeia mamária. Esses referenciais são essenciais para interpretar biomarcadores em oncologia veterinária, evitando superestimação de agressividade tumoral em fases hormonais específicas e apoiando a padronização de protocolos experimentais e diagnósticos. A inclusão deste trabalho na tese reforça o elo entre fisiologia normal e aplicação clínica, servindo de base para as análises subsequentes.

Proliferation and apoptosis dynamics of the normal canine mammary gland during the oestrous cycle evaluated by Ki-67 and Caspase-3 indexes

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Funding information

Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: 305420/2013-5; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico

Abstract

Understanding the normal physiology of the canine mammary gland (CMG) is crucial, as it provides a foundational reference for understanding canine mammary neoplasms. The relation between the Proliferation Index (PI) indicated by Ki-67 expression, along with the Apoptotic Index (AI) determined through Caspase-3 expression during the oestrous cycle, is inadequately documented in existing literature. This study seeks to offer insights into the interplay between PI and AI in the CMG across oestrous cycle phases. An extensive investigation was conducted on a diverse case series of bitches ($n = 18$). Oestrous cycle stages were determined through vaginal cytology, histological examination of the reproductive tract and serum progesterone and oestradiol concentrations. The entire mammary chain was histologically examined, and proliferation and apoptosis were assessed via double immunohistochemistry employing anti-Ki-67 and Caspase-3 antibodies. PI and AI were evaluated through a systematic random sampling approach, counting a minimum of 200 cells for each cell type. There was a significantly higher PI during early dioestrus in all mammary gland components, with a greater proportion of positive cells observed in epithelial cells compared to stromal cells. The highest PI was detected in epithelial cells within the end buds. Significant differences were found in Ki-67 labelling across the cranial mammary glands. A positive and strong correlation was noted between progesterone concentration and PI in epithelial cells. The AI remained consistently low throughout the oestrous cycle, with few differences observed across histological components. Caspase-3 labelling displayed the highest positivity in caudal mammary pairs. A negative and moderate correlation was identified between progesterone concentration and AI in interlobular mesenchymal cells. This study highlights the influence of endocrine regulation on cell proliferation indices in mammary tissue, emphasizing the need to consider these hormonal variations in toxicopathological studies involving canine mammary gland.

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KEYWORDS

histological, immunohistochemistry, mammary gland, oestrogen, progesterone

1 | INTRODUCTION

The mammary gland is an intricate anatomical structure, comprising a network of mammary ducts, lobuloalveolar units and stroma. The epithelial component includes a basal layer of myoepithelium, which possesses contractile properties, and an inner layer of luminal cells. The stroma, on the other hand, is constituted of connective tissue intermingled with blood vessels and nerves (Sorenmo et al., 2011). Interestingly, the first study of histological changes of the canine mammary gland (CMG) during the oestrous cycle was published a century ago by Marshall and Halnan (1917).

The intricate regulatory mechanisms controlling the proliferation of epithelial cells within the mammary gland are primarily orchestrated by ovarian steroid hormones, namely oestradiol and progesterone. These hormones influence cellular activities through paracrine signalling pathways (Kleinberg & Ruan, 2008). Specifically, oestradiol stimulates ductal growth, while progesterone, in conjunction with prolactin, plays a pivotal role in elongating the ductal system and lobuloalveolar development (Sorenmo et al., 2011). Yet, unlike the concise oestrous cycle observed in rats or the monthly menstrual cycle in primates, the canine reproductive system lacks a well-defined luteolytic mechanism. This leads to an extended elevation of progesterone levels, persisting between 55 and 75 days post-oestrus (Concannon, 2011). In the canine reproductive system, the corpus luteum stands as the primary source of progesterone throughout the oestrous cycle and gestation (Kowalewski et al., 2015).

Morphologically, the development of the mammary gland undergoes four distinct phases: quiescence, proliferation, differentiation and regression. Interestingly, all, except the quiescent phase can manifest during the dioestrous stage (Chandra et al., 2010; Concannon, 2011; Santos et al., 2010). The current body of literature addressing the immunohistochemical expression of proliferation and apoptosis markers in CMG predominantly centres around canine mammary neoplasms. Furthermore, most of these studies have singularly focused on a specific gland, often neglecting the varying oestrous cycle stages (Aydogan et al., 2018; Rodrigues et al., 2016). Previous research by our group revealed high proliferation rates in mammary epithelial and stromal tissues during early dioestrus (Santos et al., 2010). Yet, comprehensive detailing of the Proliferation Index (PI) and Apoptosis Index (AI) in normal CMG during the oestrous cycle was not assessed in the previous study.

Understanding the relationship between the mammary cell dynamics and the oestrous cycle is crucial for differentiating between neoplastic and physiological alterations (Christov et al., 2007). Knowing which of the proliferative activity observed in the mammary tissue is physiological or could be attributed to preneoplastic or neoplastic alterations may have clinical and therapeutic relevance.

For example, it has been demonstrated that a high proliferative index (cut-off of 40%) is significantly associated with poor overall and short disease-free survival in canine mammary carcinomas (Santos et al., 2013); yet, in such survival studies, the contribution of the physiological hormones of the oestrous cycle to the index of proliferation was not known.

Various parameters exist to evaluate proliferation, with mitotic counting via conventional haematoxylin–eosin (H&E) staining being the most straightforward. Yet, the immunohistochemical (IHC) approach using the monoclonal antibody MIB1 to detect the Ki-67 protein, a non-histone nuclear protein, is also commonly used in canine studies (Kaszak et al., 2018; Santos et al., 2010, 2013). Ki-67 expression is considered the cornerstone for assessing the PI in canine mammary tumours. Yet, standardized reference values for Ki-67 expression in normal CMG across the oestrous cycle have not been established (Carvalho et al., 2016; De Araújo et al., 2015; Lavalle et al., 2012; Nunes et al., 2018).

Apoptosis, a fundamental mechanism for ensuring cellular homeostasis, is intricately involved in various biological processes, including luteolysis and mammary morphogenesis (Reginato & Muthuswamy, 2006). This programmed cell death is primarily mediated by caspases, with Caspase-3 emerging as a pivotal indicator of apoptosis dynamics (Johnson & Bridgham, 2002; Luz et al., 2006). Given its importance, a comprehensive grasp of AI dynamics in the normal CMG provides invaluable insights into physiological versus neoplastic conditions.

With this backdrop, our study aims to achieve three core objectives: (1) delineate the PI and AI in the normal CMG across the oestrous cycle; (2) compare PI and AI across different histological components within the normal mammary gland in each oestrous cycle phase; and (3) explore potential correlations between PI and AI levels of the normal mammary tissues with serum progesterone and oestradiol concentrations.

2 | MATERIALS AND METHODS

2.1 | Case series

To fulfil our research objective, all pairs of mammary glands in different oestrous cycle phases of a heterogeneous population of intact bitches of different breeds were sampled. This sample of bitches represents the broader demographic affected by mammary gland tumours, as outlined by Santos et al. (2010). This study was approved by the Animal Experimentation Ethics Committee of the Ceará State University (UECE; protocol no. 11690648/2021).

A total of 18 bitches ($n=18$) were retrospectively enrolled in the study, all of which were euthanized for reasons unrelated to the

research and the collection of the samples was performed in the years 2002–2003. The study population consisted of a mix of mongrel and purebred adult bitches of different ages. Only non-pregnant animals displaying neither macroscopic nor microscopic mammary lesions were considered for inclusion in this study.

2.2 | Histology of reproductive system

The entire mammary chain, including the left and right first pair (M1) to the left and right inguinal pair (M5), along with the ovaries and uterus, were harvested and subsequently fixed in 10% buffered formalin. Each mammary gland was dissected at the level of the nipple, encompassing both the skin, and underlying dermal tissue. Two fragments of mammary gland measuring 1–2 cm were then subjected to routine processing for paraffin embedding.

For histological analysis, sections of 3 µm thickness were obtained from each paraffin block. One section was subjected to H&E staining and subsequently submitted for microscopic evaluation. It should be noted that the number of nipples varied among the dogs, and a transversal section was performed during macroscopic trimming of the entire mammary gland solely when a nipple was discernible (thus, the identification of each mammary gland pair was performed by the identification of the nipple).

2.3 | Determining the oestrous cycle phase

An initial classification of the oestrous cycle stage was conducted through a multi-faceted approach. Vaginal cytology, performed immediately after the euthanasia, employing air-dried smears stained with Hemacolor (Merck, Darmstadt, Germany), in conjunction with histological examination of the ovaries, uterus and vagina, as previously described (Santos et al., 2010). Following this preliminary morphological categorization, the animals were further stratified due to their serum progesterone and 17-β oestradiol concentration levels. Serum samples were taken immediately before euthanasia and stored at -80°C. Serum concentrations of progesterone and 17-β oestradiol were subsequently quantified by a chemiluminescent method (Immulite assay; Diagnostic Products Corporation, Deerfield, IL, USA), as previously described (Kustritz, 2012; Santos et al., 2010).

Anoestrus was identified by low cellularity, non-cornified cells and basal progesterone levels below 0.5 ng/mL. Proestrus was characterized by intermediate and superficial cells, red blood cells in cytology, proliferative endometrial glands histologically and progesterone levels below 0.5 ng/mL. Oestrus was marked by over 80% cornified cells in vaginal smears, developing corpora lutea, and progesterone levels between 1.0 and 10 ng/mL. Early dioestrus showed a shift to non-cornified cells and polymorphonuclear cells with progesterone concentrations of 1.0–10 ng/mL, while late dioestrus exhibited intermediate cells dominance, formed corpora

lutea, secretory endometrium and progesterone levels between 15 and 60 ng/mL.

2.4 | Immunohistochemistry

The normal mammary paraffin blocks were retrieved from the archives of the research group from ICBAS, University of Porto and sections of 3 µm thickness were obtained from each paraffin block. The PI activity and AI were assessed by double immunohistochemistry using Ki-67 monoclonal antibody [clone MIB-1 (Dako, Glostrup, Denmark), dilution 1:100] and Cleaved Caspase-3 polyclonal antibody [Asp175 (Cell Signaling Technology, Beverly, MA, USA), dilution 1:100], respectively. Heat-induced antigen retrieval was performed in a pressure cooker, using a citrate buffer (pH 6.0) maintained for 3 min after maximum pressure was reached. A polymer-based immunostaining kit (Novolink Max Polymer Detection System, Novocastra, Newcastle upon Tyne, England) was used. The Ki-67 signal was evidenced by brown staining within the nuclei with the 3,3'-diaminobenzidine chromogen, and the Caspase-3 signal evidenced by a red staining in the cytoplasm with the aminoethyl carbazole chromogen. The antibody of the first reaction (MIB-1) was eluted from the slides by immersing in a glycine buffer (pH 2.2), before the second labelling (Caspase-3) was started. Positive (reactive canine lymph nodes) and negative controls were included in each immunohistochemical run. All sections were counterstained with Mayer's haematoxylin, washed in water and coverslipped.

To count the immunostained cells for MIB-1 and anti-caspase 3 antibodies, the mammary gland cell populations were sorted into epithelial cells, intralobular (loose connective tissue surrounding the epithelium) and interlobular (dense supportive connective tissue) stromal cells. The epithelial component was further subdivided into epithelial luminal cells of the ducts, epithelial luminal cells of intralobular structures (mainly secretory, inactive or regressive alveoli, depending on the oestrous phase), basal cells (those closed to the basement membrane) and epithelial cells of terminal end buds (solid aggregates of cells in the walls of the ductal branching structures).

The slides of the double immunohistochemistry were scanned in the Olympus VS110 slide scanner, equipped with a BX61VS microscope and a XC10 digital camera (Olympus, Japan). Counting of Ki-67 and Caspase-3 immunostained cells were performed following a systematic random sampling design. Random points were selected within the mammary tissue and microphotographs were taken at 400x magnification in consecutive constant steps from the digital slide. A grid with eight frames (with inclusion and exclusion lines) was overlaid to each microphotograph and the counting was performed in the randomly selected frames. With this approach, at least 200 cells of all the cell types were evaluated in each mammary gland pair by the same observer (BQP) blinded to the oestrous cycle phase and to the hormonal levels. This approach is known to ensure

efficient and reliable sampling (Gundersen et al., 1988). The percentage of Ki-67 (PI) and Caspase-3 (AI) positive cells was calculated by the number of positive cells divided by the total number of cells assessed (Hvid et al., 2012, 2020).

2.5 | Statistical analysis

Statistical analysis was performed using R software, version 4.0.2 (R® Foundation for Statistical Computing, Austria). Data were expressed as mean and standard deviation. The initial statistical models included five explanatory variables: oestrous phase, mammary pair gland, histological structure, progesterone and 17- β oestradiol levels. The normality and homoscedasticity of the results were evaluated, using the Shapiro–Wilk and Levene tests, respectively. For comparison of the averages, the one-way ANOVA test was used, followed by the Tukey test as a post-hoc. If data distribution was not normal, Kruskal–Wallis test was used to compare values. For correlation, due to the small sample number and the repetition of results, Kendall's correlation coefficient was chosen. Using backwards selection, non-significant variables were removed one at the time, until only significant explanatory variables remained. Statistical tests were considered significant at a level set at 5% ($p < .05$).

3 | RESULTS

3.1 | Oestrous cycle categorization

Following the oestrous cycle determination analyses, the study population was categorized into five distinct groups: anoestrus ($n=5$), proestrus ($n=3$), oestrus ($n=4$), early dioestrus ($n=3$) and late dioestrus ($n=3$). Subsequent quantitative evaluations of Ki-67 and Caspase-3 expression were conducted in each slide corresponding to each mammary gland pair ranging from 3 to 5 pairs.

3.2 | Immunostaining patterns

Immunostaining analysis revealed distinct patterns for Ki-67 and Caspase-3. Ki-67 exhibited nuclear staining with different intensity (all ranges of intensities were considered as positive staining) characterized by a granular labelling pattern associated with the nuclei. Notably, staining within the nucleoli was more homogeneous and exhibited intense reactivity. In contrast, Caspase-3 immunoreactivity manifested as cytoplasmic staining, displaying a granular, punctuated distribution (Figures 1 and 2).

3.3 | Ki-67 expression dynamics

The mammary PI index (%) showed increased values in oestrus (6.1 ± 3.7), a peak during early dioestrus (28.8 ± 9.3) and a decrease in late dioestrus (3.3 ± 2.6). The lowest PI values were evident during proestrus (1.4 ± 1.8) and anoestrus (1.9 ± 3.0). In terms of Ki-67 positivity, a higher proportion of positive cells was discerned in the epithelial compared to stromal cells. When it comes to the histological substructure of the mammary gland during early dioestrus, the epithelial cells within the end buds (42.3 ± 11.2) demonstrated the most elevated PI, followed by cells in basal location (33.1 ± 10.9) and then by the epithelial cells of the intralobular structures (27.6 ± 14.6). In the statistical analysis, only early dioestrus presented a significant higher number ($p = .002$) of PI cells in all the components of the gland (intralobular and interlobular stroma, basal cells, epithelial luminal cells of intralobular structures, epithelial luminal cells of the ducts and cells of terminal end buds; Figure 3).

3.4 | Spatial variations in Ki-67 labelling

Subtle yet statistically significant variations were noted in Ki-67 labelling among the examined mammary gland pairs during each phase

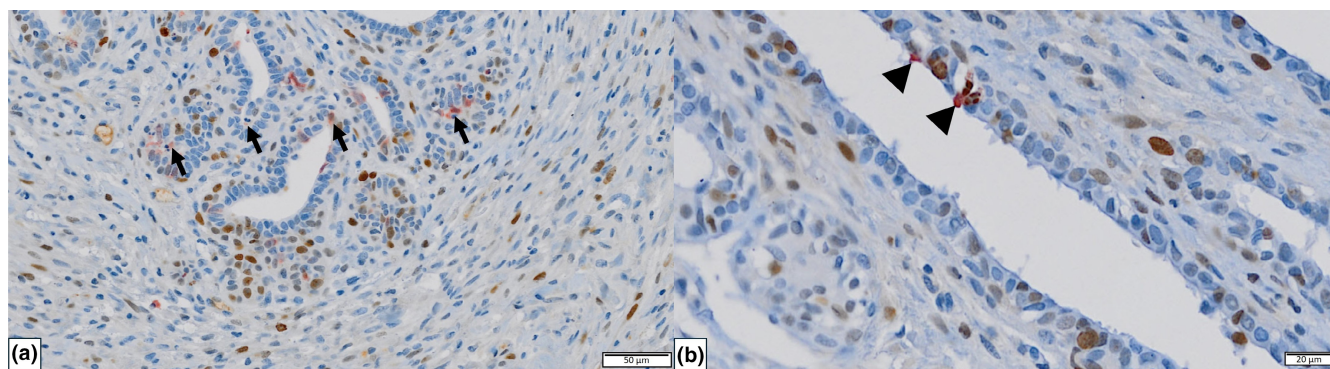


FIGURE 1 Normal canine mammary gland during early dioestrus. Brown nuclei positive to Ki-67 antibody (MIB-1) were observed in epithelial structures and in the stroma. Apoptotic cells, detected by a red colour in the cytoplasm (indicative of immunolabelling with anti-caspase-3). (a) Ducts, ductal branches and end buds were surrounded by a highly cellular loose connective tissue. Note more apoptotic cells in the epithelial structures (arrows) than in the stroma. (b) Ducts and ductal branches surrounded by fusiform connective cells. Two apoptotic cells were observed in the luminal lining of the large duct (arrowhead). Diaminobenzidine and Aminoethylcarbazole chromogens with a haematoxylin counterstain (a and b). Bar = 50 μ m (a) and 20 μ m (b).

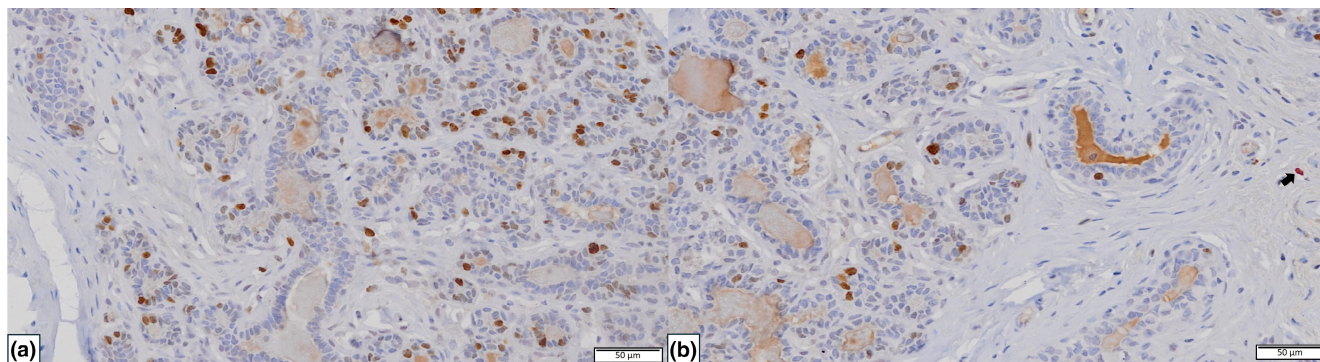


FIGURE 2 Normal canine mammary gland in late dioestrus. Intralobular duct and secretory alveoli were observed. Positive nuclei to Ki-67 were identified mainly in the luminal and basal cells of the alveoli, with few connective cells being also positive. In this oestrous cycle phase, background staining in the secretion was frequently present, but this did not mask the specific immunolabelling to Ki-67 and Caspase-3. (a) In this field, none of the cells were positive to Caspase-3 (no red cytoplasmic staining was observed). (b) In this field, only one positive cell to caspase-3 was observed in the stroma (arrow). Diaminobenzidine and Aminoethylcarbazole chromogens with a haematoxylin counterstain (a and b). Bar = 50 µm (a and b).

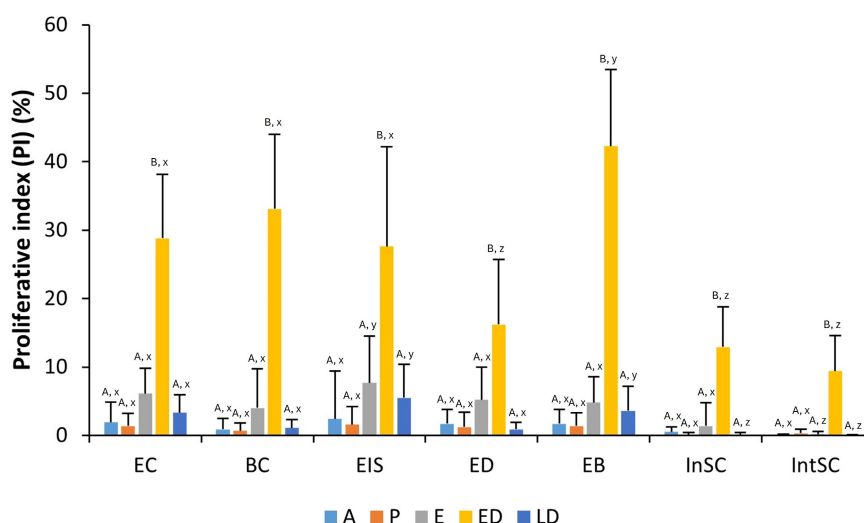


FIGURE 3 Proliferative index (PI; %) obtained by immunohistochemical evaluation of MIB antibody (detection of Ki-67) staining in different histological components of the normal mammary gland of bitches in different oestrous cycle phases. Data are presented as mean and standard deviation. A, anoestrus; BC, total basal cells; E, oestrus; EB, end bud cells; EC, total epithelial cells; ED, early dioestrus; ED, epithelial cells of ducts; EIS, epithelium of the intralobular structures; InSC, intralobular stroma cells; IntSC, interlobular stroma cells; LD, late dioestrus; P, proestrus. Different capital letters represent statistical differences within oestrous cycle phases ($p < .05$). Different lowercase letters represent statistical differences between oestrous cycle phases ($p < .05$).

of the oestrous cycle. Notably, during the initial dioestrous phase, characterized by the highest PI across all histological components, a higher Ki-67 index was evident in the cranial mammary glands ($P1 = 32.3 \pm 14.3$; Table 1).

3.5 | Hormonal correlations with proliferative index

A moderate and statistically significant correlation was observed between the concentration of progesterone and the PI in almost all the histological components and cell types, except end bud and

mesenchymal cells of the intralobular stroma. The Kendall tau-b correlation coefficient (τ) was high in epithelial cells (0.6, $p = .008$). In contrast, no significant associations were detected between 17β oestradiol concentration and the PI (Table 2).

3.6 | Apoptosis dynamics

Apoptosis was present in all cell types of the CMG throughout the oestrous cycle. Yet, the AI, represented by the ratio between the number of Caspase-3 positive cells and the total of cells, remained very low ($AI < 1\%$ in the stroma and in the epithelium) throughout the

	A	P	E	ED	LD
M1	1.2 ± 0.8 ^A	2.4 ± 1.6 ^A	3.5 ± 2.9 ^A	32.3 ± 14.3 ^A	0.3 ± 0.2 ^A
M2	1.5 ± 1.0 ^A	0.4 ± 0.2 ^B	5.9 ± 3.9 ^A	25.1 ± 12.3 ^{AB}	2.0 ± 2.4 ^A
M3	0.9 ± 0.7 ^A	0.8 ± 0.5 ^{AB}	3.8 ± 2.7 ^A	24.8 ± 11.0 ^{AB}	1.9 ± 2.0 ^A
M4	2.5 ± 2.9 ^A	0.7 ± 0.7 ^B	3.8 ± 2.5 ^A	16.1 ± 9.8 ^B	2.8 ± 2.7 ^A
M5	1.4 ± 1.3 ^A	0.8 ± 0.9 ^B	2.8 ± 2.4 ^A	23.3 ± 12.8 ^{AB}	3.2 ± 3.4 ^A

Note: Different capital letters in the same column represent statistical differences with *p* value <.05 in the same oestrous phase.

Abbreviations: A, anoestrus; E, oestrus; ED, early dioestrus; LD, late dioestrus; M1, cranial thoracic mammary gland; M2, caudal thoracic mammary gland; M3, cranial abdominal mammary gland; M4, caudal abdominal mammary gland; M5, inguinal mammary gland; P, proestrus.

TABLE 1 Mean and SD of the proliferative index (PI; %) determined by immunohistochemical evaluation with MIB antibody (detection of Ki-67) in the normal mammary gland chain pairs of bitches in the different oestrous cycle phases.

TABLE 2 Kendall's correlation value (*r*) between hormonal dosage of serum progesterone and 17-β oestradiol and the proliferative index (PI; %), determined by immunohistochemical evaluation with MIB antibody (detection of Ki-67), in the different histological structures the normal mammary gland of bitches.

	EC	BC	EIS	ED	EB	InSC	IntSC
Progesterone (ng/mL)	0.6* (<i>p</i> = .008)	0.5* (<i>p</i> = .02)	0.5* (<i>p</i> = .02)	0.5* (<i>p</i> = .02)	0.4 (<i>p</i> = .06)	0.3 (<i>p</i> = .1)	0.5* (<i>p</i> = .04)
17-β oestradiol (pg/mL)	0.2 (<i>p</i> = .3)	0.2 (<i>p</i> = .3)	0.2 (<i>p</i> = .2)	0.2 (<i>p</i> = .4)	0.08 (<i>p</i> = .7)	0.04 (<i>p</i> = .9)	0.2 (<i>p</i> = .5)

Abbreviations: BC, total basal cells; EB, end bud cells; EC, total epithelial cells; ED, epithelial cells of ducts; EIS, epithelial cells of the intralobular structures; InSC, intralobular stromal cells; IntSC, interlobular stromal cells.

*Represents statistical differences with *p* value <.05.

oestrous cycle, with no significant difference between the oestrous phases. Statistically significant differences (*p* = .03) were exclusively observed among histological components during the late dioestrus, with a high AI in the intralobular stroma (1.1 ± 1.0) in comparison with the other histological structures and components (Figure 4). A trend to a high AI in the caudal mammary pairs was also noted, particularly during the oestrus and early dioestrus, irrespective of the histological component (Table 3).

3.7 | Hormonal correlations with apoptosis index

Regarding the correlation analysis, a negative moderate and borderline statistically significant association was observed between the concentration of progesterone and the AI of the interlobular stromal cells (Kendall tau-b (*r*) -0.4, *p* = .05). Yet, no significant associations were found between 17-β oestradiol concentration and AI (*p* > .05; Table 4).

4 | DISCUSSION

In this study, we determined the PI and AI of the normal mammary gland in 18 intact bitches of various breeds and ages. The PI and AI were assigned using a systematic random sampling approach and a minimum of 200 cells from different histological components were evaluated in each mammary gland pair. The evaluation was performed by the same observer, blinded to the oestrous cycle phase of the bitches, in order to ensure consistency. The quantification of

Ki-67 and Caspase-3 indexes in each mammary gland pair and histological component provides baseline data for future comparisons with PI and AI levels associated with the most common pathological conditions of the canine mammary gland, particularly neoplastic lesions.

The adoption of random sampling is considered fundamental for precisely estimating the proportion of proliferating cells within a tissue section (Gundersen et al., 1988). The number of total counted cells is also relevant for the precision of the estimation of indices and herein more than 200 cells of each subtype were considered. This threshold was set following Hvid et al. (2020), who suggested that increasing the number of visual fields to 400 and the cell count to 200 within the rat mammary gland would not significantly alter the magnitude or variability of PI values.

Normal mammary gland microstructure has been extensively investigated among rodents (Hvid et al., 2012, 2020; Imaoka et al., 2012). In dogs, much less studies existed to date, but all of them demonstrated notable morphological changes within the mammary gland during the ovarian cycle (Chandra et al., 2010; Santos et al., 2010). The cellular responses in hormone-dependent organs including the mammary gland are orchestrated by steroid hormones, as well as prolactin and growth hormone (Michel et al., 2012; Spoerri et al., 2015). Remarkably, in the CMG only the PI seemed to be influenced by the oestrous cycle phase, with significantly higher values in early dioestrus. This phase, characterized by heightened progesterone levels resulting from the functional corpora lutea activity (Vanderperren et al., 2018), exhibited notable impacts on the PI of both epithelial and stromal populations. Progesterone emerges as a significant mitogen for the mammary gland, acting either directly on cells expressing

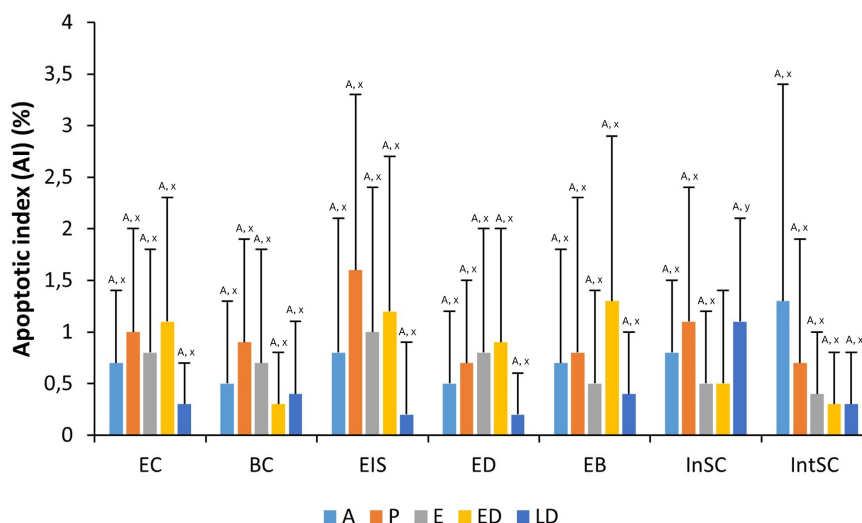


FIGURE 4 Apoptotic index (AI; %) obtained by immunohistochemical evaluation of cleaved Caspase-3 polyclonal antibody staining in different histological regions of the normal mammary gland of bitches in different oestrous cycle phases. Data are presented as mean and standard deviation. A, anoestrus; BC, total basal cells; E, oestrus; EB, end bud cells; EC, total epithelial cells; ED, early dioestrus; ED, epithelial cells of ducts; EIS, epithelium of the intralobular structures; InSC, intralobular stroma cells; IntSC, interlobular stroma cells; LD, late dioestrus; P, proestrus. Different capital letters represent statistical differences within oestrous cycle phases ($p < .05$). Different lowercase letters represent statistical differences between oestrous cycle phases ($p < .05$).

TABLE 3 Mean and SD of the apoptotic index (AI; %), determined by immunohistochemical detection of cleaved Caspase-3 in the normal mammary gland chain pairs of bitches in different oestrous cycle phases.

	A	P	E	ED	LD
M1	1.0 ± 0.7 ^A	1.7 ± 1.2 ^A	0.4 ± 0.6 ^A	0.6 ± 0.2 ^A	0.2 ± 0.2 ^A
M2	0.4 ± 0.4 ^A	0.3 ± 0.4 ^B	0.2 ± 0.2 ^A	0.2 ± 0.1 ^A	0.6 ± 0.4 ^A
M3	0.7 ± 0.5 ^A	1.1 ± 0.4 ^{AB}	0.8 ± 0.5 ^{AB}	0.9 ± 0.5 ^{AB}	0.4 ± 0.5 ^A
M4	0.6 ± 0.2 ^A	1.1 ± 0.6 ^{AB}	0.6 ± 0.4 ^A	0.9 ± 0.8 ^{AB}	0.2 ± 0.5 ^A
M5	0.8 ± 0.5 ^A	1.1 ± 0.8 ^{AB}	1.7 ± 0.7 ^B	1.6 ± 1.1 ^B	0.7 ± 0.5 ^A

Note: Different capital letters in the same column represent statistical differences with p value $< .05$ in the same oestrous phase.

Abbreviations: A, anoestrus; E, oestrus; ED, early dioestrus; LD, late dioestrus; M1, cranial thoracic mammary gland; M2, caudal thoracic mammary gland; M3, cranial abdominal mammary gland; M4, caudal abdominal mammary gland; M5, inguinal mammary gland; P, proestrus.

TABLE 4 Kendall's correlation value (τ) between levels of serum progesterone and 17- β oestradiol and the apoptotic index (AI; %), determined by immunohistochemical evaluation with cleaved Caspase-3 polyclonal antibody in the different histological structures the normal mammary gland of bitches.

	EC	BC	EIS	ED	EB	InSC	IntSC
Progesterone (ng/mL)	-0.2 ($p = .4$)	0 ($p = 1$)*	-0.2 ($p = .4$)	0 ($p = 1$)	-0.2 ($p = .3$)	-0.4 ($p = .07$)	-0.4 ($p = .05$)
17- β oestradiol (pg/mL)	-0.03 ($p = .9$)	0.21 ($p = .4$)	-0.1 ($p = .5$)	-0.1 ($p = .6$)	-0.1 ($p = .5$)	-0.01 ($p = .9$)	-0.2 ($p = .3$)

Abbreviations: A, anoestrus; BC, total basal cells; E, oestrus; EB, end bud cells; EC, total epithelial cells; ED, early dioestrus; ED, epithelial cells of ducts; EIS, epithelial cell of the intralobular structures; InSC, intralobular stromal cells; IntSC, interlobular stromal cells; LD, late dioestrus; P, proestrus.

progesterone receptors or indirectly influencing non-expressing cells through the paracrine mechanism involving the receptor activator of nuclear factor κ B ligand (RANKL; Beleut et al., 2010).

In rats, the PI of the mammary epithelium remains low during the follicular stage and experiences a notable increase during the luteal stage (Hvid et al., 2012, 2020). In the case of bitches, corroborated by other reports, qualitative evidence of glandular and/or stromal proliferation is predominantly discernible during the early

dioestrous phase (Chandra et al., 2010; Rehm et al., 2007; Santos et al., 2010; Vanderperren et al., 2018). Subsequently, the gland undergoes regression in late dioestrus and persists in a predominantly quiescent state during anoestrus, proestrus and oestrus, with no relevant qualitative differences.

Our investigation also reveals a discernible impact of the histological region on the PI, with the highest rates being observed in the end buds that correspond to points for future ductal branching.

This finding is in accordance with our previous morphological study in which mitotic figures were frequently found in ductal branches during early dioestrous phase (Santos et al., 2010). In female rats, conflicting results regarding the distribution of the Ki-67 labelled cells were reported, with some authors describing a random distribution (Schedin et al., 2000), while others reported that the proliferative cells were primarily localized in end buds (Hvid et al., 2012).

In our study, we observed a moderate but significant correlation between serum progesterone levels and the PI across almost all evaluated histological components. Conversely, no significant correlation was detected between serum oestradiol levels and the PI. It is tempting to speculate that, similarly to other species, in bitches the apparent delay between the peak concentrations of oestradiol and the peak of PI is attributable to the requirement of paracrine mechanisms for the mammary proliferation burst. This hypothesis is sustained by the fact that most of the proliferating cells do not express oestradiol (ER) and progesterone (PR) receptors (Clarke, 2003; Mallepell et al., 2006). The occurrence of the peak of PI in canine mammary gland during the early dioestrus indicates that all the hormonal and paracrine stimuli necessary to the proliferation have already occurred.

In the dog mammary gland, ER levels are low during proestrus through early dioestrus, peak during late dioestrus and subsequently decline to low levels during anoestrus. Meanwhile, PR concentrations, although low, are slightly higher than those of ER during proestrus and oestrus. PR increases and peaks in tandem with ER during early to mid-dioestrus, remaining elevated during anoestrus (Donnay et al., 1995; van Garderen & Schalken, 2002). Additionally, it has been established that low concentrations of oestrogens can stimulate the expression of PR, and the effectiveness of progesterone actions is dependent on the presence of oestrogen (Clarke, 2003; Pompei et al., 2005). This means that the peak of cell PI is more likely to be observed following exposure to both oestrogen and progesterone, which in bitches occurs in dioestrus. Previous evidence in rat models suggested that a substantial influx of cells into the S-phase of the cell cycle occurred in luteal phase (hormonally equivalent to the early dioestrus in bitches; Schedin et al., 2000). Interestingly, our findings of increased number of proliferative cells in dioestrus correlates well with the increased blood influx (as detected by ultrasonography) in the canine mammary gland in the same oestrous phase (Vanderperren et al., 2018).

In the present study, apoptosis was observed across all cell types and histologic components of CMG throughout the oestrous cycle. Nevertheless, the incidence of apoptosis was very low (AI < 1%) in both the epithelium and stroma, with the highest number of apoptotic cells being identified during proestrus. Yet, the differences among the other oestrous phases did not reach statistical significance. Similar results were reported in female rats, where no statistically significant relation between the AI and the oestrous cycle was found. Comparatively, human mammary tissue exhibited a higher number of morphologically identified apoptotic cells during the early follicular stage, which also declined during the late follicular and early luteal stages, and subsequently increased during the menstrual stage of the cycle (Anderson et al., 1982; Ferguson & Anderson, 1981). Herein, no

significant correlation was established between the AI (determined based on caspase-3 immunolabelling) and serum oestrogen. Yet, a borderline negative correlation was noted with the progesterone concentrations. Additionally, an intriguing observation in this study was the trend to a higher AI in the caudal mammary pairs during the oestrus and early dioestrus. Histologically a greater number of ductal branches and lobules were described in the caudal abdominal and inguinal mammary glands compared to the thoracic mammary glands (Santos et al., 2010). This observation potentially justifies the higher number of cells undergoing apoptosis before the progression to the subsequent phases of regressive and quiescent features.

Our data indicate that apoptosis, recognized as a crucial process in reproductive phenomena, such as the duration of corpus luteum (Stornelli et al., 2020), may also have a role in the extensive cellular alterations of the CMG and can be regulated in this context by different mechanisms and not exclusively by Caspase-3.

In fact, accumulating evidence suggests that cell death can follow a programmed pattern, occurring independently of caspase activation (Tait & Green, 2008). Various models of programmed cell death have been suggested, including autophagy, paraptosis, mitotic catastrophe and caspase-independent cell death (CICD) pathways. Even though CICD pathways have various enzymes involved in their cascades, they frequently share common features. At times, the same stimuli can enable these two pathways at the same time (Cregan et al., 2004). Further studies are required to elucidate the role of apoptosis in the oestrous cycle-dependent morphological alterations in the normal CMG, particularly the regression of lobuloalveolar structures. For depth exploration, alternative apoptotic markers should be assessed in normal CMG.

Ultimately, increased cell proliferation can be a normal physiological response to hormonal stimuli, but can also be a feature of preneoplastic or neoplastic lesions (Elcombe et al., 2002). In canine mammary neoplasms, a negative correlation between active caspase-3 and Ki-67 emphasizes the intricate interplay between proliferation and apoptosis dynamics (Rodrigues et al., 2016). Knowing the dynamics of PI and AI in the normal CMG can be useful for identifying subtle tumour-related alterations or those alterations occurring in toxicopathological studies.

5 | CONCLUSION

In summary, our study establishes the existence of endocrine regulation of the proliferation activity of the normal mammary canine gland, notably displaying a substantial increase during early dioestrus when compared to other oestrous phases. Apoptosis, as assessed by caspase-3 immunolabelling, remains a rare phenomenon in the normal CMG, although the plausible influence of endocrine factors on apoptosis suggests potential variations in the AI among mammary gland pairs, particularly in specific oestrous cycle phases. These findings emphasize the significance of accounting these hormonal fluctuations in both PI and AI assessments, when examining pathological alterations in canine mammary gland. Such insights may prove valuable for

veterinary pathologists aiding the intricate task of distinguishing between normal oestrous cycle-dependent mammary characteristics and those associated with the carcinogenesis process.

AUTHOR CONTRIBUTIONS

Breno Pinheiro provided the investigation, formal analysis and writing of the original draft. Emanuel Cavalcante provided the investigation, formal analysis and writing of the original draft. Célia Lopes provided the methodology, investigation and final revision of the manuscript. Ricardo Marcos provided the methodology, investigation and final revision of the manuscript. Lúcia Silva provided conceptualization, supervision and final revision of the manuscript. Marta Santos collected the samples, provided conceptualization, methodology, writing and final revision of the manuscript. Augusto Faustino provided conceptualization, methodology, supervision, funding and final revision of the manuscript.

ACKNOWLEDGEMENTS

BQP was supported by the ICBAS mobility program (Student Mobility for Traineeships). The authors also thank the following development agencies for financial support: the National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq) through individual research grants to LDMS (grant number 305420/2013-5); the Brazilian Federal Agency for the Support and Evaluation of Graduate Education (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES); and the Ceará State Support for Scientific and Technological Development Foundation (Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico - FUNCAP) for granting a scholarship during this study.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

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

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How to cite this article: Pinheiro, B. Q., Cavalcante, F. E. P., Lopes, C. C. C., Marcos, R. J. P. C., da Silva, L. D. M., dos Santos, M. S. A., & Faustino, A. M. R. (2024). Proliferation and apoptosis dynamics of the normal canine mammary gland during the oestrous cycle evaluated by Ki-67 and Caspase-3 indexes. *Reproduction in Domestic Animals*, 59, e14655. <https://doi.org/10.1111/rda.14655>

7 CAPÍTULO II

Serum CA 15-3 and immunohistochemical Ki-67 and COX-2 expression in canine mammary neoplasms: biomarkers for prognosis and survival prediction

Expressão sérica de CA 15-3 e imuno-histoquímica de Ki-67 e COX-2 em neoplasias mamárias caninas: biomarcadores para prognóstico e predição de sobrevida

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Dados do manuscrito: *Veterinary and Comparative Oncology* (Wiley). **FI:** 2,61. **Qualis:** A1. Status: manuscrito em submissão, versão inicial submetido à publicação em 17/06/2025.

Este estudo prospectivo incluiu 54 cadelas com neoplasias mamárias e 12 controles saudáveis, avaliando CA 15-3 sérico em série e a expressão tecidual de Ki-67 e COX-2. O CA 15-3 foi indetectável nos controles e mais elevado nos tumores malignos, com correlações robustas com estadiamento clínico, grau histológico e número de nódulos; a análise ROC indicou ponto de corte de 3,01 IU/mL com AUC de 0,92 para distinguir malignos de benignos. O Ki-67 correlacionou-se ao grau, enquanto a COX-2 mostrou desempenho diagnóstico limitado, com AUC de 0,44. Em conjunto, biomarcadores séricos e teciduais melhoraram a estratificação prognóstica e sustentam decisões terapêuticas, apoiando o uso de painéis integrados na clínica de neoplasias mamárias caninas.

TITLE

Serum CA 15-3 and immunohistochemical Ki-67 and COX-2 expression in canine mammary neoplasms: biomarkers for prognosis and survival prediction

RUNNING HEAD

CA 15-3, Ki-67, and COX-2 in canine mammary neoplasms

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ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Clinical Pathology Laboratory of the Sylvio Barbosa Cardoso Veterinary Hospital, Faculty of Veterinary Medicine, State University of Ceará (UECE), for performing the hematological and biochemical analyses of the patients included in this study. We are also grateful to the Comparative Pathology Laboratory (LPC), Institute of Biomedical Sciences, Federal University of Minas Gerais (UFMG), for the histopathological processing of neoplastic samples, with special thanks to Professor Geovanni Dantas Cassali for his valuable support. We further acknowledge veterinarians Fabiane Gruhn Roth and Francisco Sávio de Moura Martins, from Vetex Laboratory, for their collaboration and for conducting the CA 15-3 biomarker assays. Immunohistochemical analyses for Ki-67 and COX-2 were carried out at the Veterinary Pathology Laboratory (LABPATVETUP), Department of Pathology and Molecular Immunology, School of Medicine and Biomedical Sciences Abel Salazar (ICBAS), University of Porto, where we especially thank Ms. Maria de Fátima dos Santos Faria de Carvalho for her technical assistance.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

This work was supported by the National Council for Scientific and Technological Development (CNPq), grant number #305420/2013-5.

AUTHOR CONTRIBUTIONS

Breno Queiroz Pinheiro: conceptualization, data curation, writing original draft.

Marcely Braga de Albuquerque, Francisco Emanuel Pinheiro Cavalcante, Isabela Reis Barroso do Nascimento: methodology, investigation. Fernanda Rezende Souza: histopathological analysis, validation. Augusto Manuel Rodrigues Faustino and Lúcia Daniel Machado da Silva: supervision, writing, review & editing.

ABSTRACT

Canine mammary neoplasms (CMNs) are among the most frequent neoplasms in intact female dogs and represent a valuable spontaneous model for comparative oncology. This study aimed to evaluate the prognostic value of serum CA 15-3 and immunohistochemical (IHC) expression of Ki-67 and COX-2 in CMNs. Fifty-four female dogs with histologically confirmed CMNs and twelve healthy controls were enrolled. Serum CA 15-3 levels were measured preoperatively and at 21 and 90 days after mastectomy, while Ki-67 and COX-2 were assessed in tissue samples. CA 15-3 was undetectable in controls and significantly higher in malignant than in benign neoplasms ($p < 0.05$), showing strong correlations with clinical stage ($s = 0.59$), histological grade ($s = 0.64$), and the number of nodules ($s = 0.42$). Levels remained elevated in advanced stage cases even after surgery. ROC curve analysis identified a CA 15-3 cut-off of 3.01 IU/mL (AUC = 0.92) to differentiate malignancy. Ki-67 correlated with histological grade ($p = 0.41$) but showed limited prognostic accuracy (AUC = 0.63). COX-2 expression showed no significant associations and exhibited poor discriminatory capacity (AUC = 0.44). Survival analyses revealed a trend toward reduced survival in dogs with higher CA 15-3, Ki-67 or COX-2 expression. Principal component analysis indicated clustering of aggressive neoplasms according to biomarker profile. The combination of serum and tissue biomarkers improved prognostic stratification and may guide therapeutic decision-making in veterinary oncology. These findings support the growing role of biomarker panels in the clinical management of CMNs and emphasize their potential translational relevance to human breast cancer.

KEYWORDS:

Immunohistochemistry; Prognostic stratification; Serum biomarkers; Surgical follow-up; Neoplastic burden; Veterinary oncology

INTRODUCTION

Canine mammary neoplasms (CMNs) are the most frequently diagnosed neoplasms in intact female dogs, accounting for an incidence rate of up to 42% and a prevalence of up to 70% for highly aggressive lesions.¹ Due to their biological, histological, and molecular similarities to human breast cancer, CMNs are considered an important model for comparative oncology studies.^{2,3} Identifying prognostic and predictive biomarkers in CMNs is critical for guiding treatment decisions and improving outcomes.^{4,5}

Among these biomarkers, cancer antigen 15-3 (CA 15-3), Ki-67, and cyclooxygenase-2 (COX-2) have emerged as candidates of particular interest. CA 15-3, a circulating mucin-type glycoprotein primarily derived from the mucin 1 gene product, traditionally used in human breast neoplasms (HBNs) medicine for disease monitoring and prognostic assessment.⁶ In veterinary medicine, although still underexplored, CA 15-3 has shown potential as a non-invasive biomarker for disease aggressiveness

and neoplasm burden.^{7,8} Recent evidence suggests that elevated CA 15-3 serum levels correlate with advanced clinical staging, diameter, lymph node involvement, and metastatic progression in CMNs.⁸⁻¹⁰ These findings highlight CA 15-3 as a clinically valuable serum biomarker in the veterinary oncology setting, especially when applied in conjunction with histopathological evaluation and other diagnostic tools.

Ki-67 is a nuclear protein associated with cell proliferation, expressed during all active phases of the cell cycle (G1, S, G2, and M) and absent in quiescent cells (G0). This specific expression profile makes Ki-67 a valuable biomarker for assessing the proliferative activity of CMNs cells.¹¹ High Ki-67 index values have been linked to high-grade neoplasms, metastasis, and reduced overall survival in CMNs.¹¹⁻¹⁵ Furthermore, studies on normal mammary gland physiology have shown hormonal modulation of proliferative activity, emphasizing the relevance of Ki-67 as both a physiological and pathological marker.¹⁶

COX-2, an inducible enzyme involved in prostaglandin synthesis, is upregulated in response to inflammatory and neoplastic stimuli, contributing to key hallmarks of cancer such as sustained proliferation, angiogenesis, and immune evasion in several malignancies, including CMNs.^{17,18} Overexpression of COX-2 in CMNs has been associated with high histological grade, lymph node metastasis, and reduced survival, suggesting its value as both a prognostic marker and a potential therapeutic target.¹⁸⁻²⁰ The overexpression of COX-2 in malignant tissues compared to normal or benign mammary tissue further supports its application in distinguishing aggressive neoplastic phenotypes in veterinary oncology.^{21,22}

Despite the growing evidence of the individual prognostic relevance of these biomarkers, there is a lack of studies investigating their integrated prognostic value in CMNs. This study aims to evaluate the association between serum CA 15-3 levels and immunohistochemical (IHC) expression of Ki-67 and COX-2 in CMNs and to determine their prognostic significance through survival analysis.

METHODS

This study was approved by the animal experimentation ethics committee of the institution (protocol no. 11690648/2021), and all dog owners who agreed to participate in the current study received proper orientation and signed a consent form. The data supporting the findings of this study are available upon request. No cell lines were used in this study.

1. Study Design and Animal Selection

A total of 84 female dogs with CMNs were initially screened prospectively for inclusion in this study. However, some were excluded due to incomplete follow up, previous oncological treatments, or insufficient sample quality, resulting in a final cohort of 54 bitches with CMNs that met all inclusion criteria. The selected dogs were divided into six groups, including a control group (12 clinically healthy Australian Shepherd bitches from a commercial kennel, which underwent general clinical evaluation and laboratory testing to confirm their absence of CMNs). The neoplasm-bearing dogs were subdivided as follows:

- Benign (B): Dogs with histopathologically confirmed only benign CMNs (9/54).
- Stage I: Malignant CMNs carcinomas measuring ≤ 3 cm in diameter, with no evidence of lymph node or distant metastasis (11/54).
- Stage II: Malignant CMNs carcinomas measuring 3-5 cm in diameter, without lymph node or distant metastasis (13/54).

- Stage III: Malignant CMNs carcinomas measuring >5 cm in diameter, without evidence of metastasis (11/54).
- Stage IV: Malignant CMNs carcinomas of any size with confirmed regional lymph node metastasis (10/54).

The subdivision followed the TNM system and the guidelines of the World Health Organization (WHO).²³

2. Clinical, Surgical and Histopathological Evaluation

Each dog underwent a complete clinical examination, including lymph node palpation, neoplasm measurement, and assessment for ulceration or necrosis. Blood samples were collected for hematological and biochemical analysis. Thoracic radiographs (three projections) and abdominal total ultrasound were performed to evaluate metastatic spread. A variety of procedures were established based on CMNs size, fixation to the surrounding tissue, number of lesions, as well as regional lymph nodes (inguinal and/or axillary).²⁴ Histopathological classification was performed using hematoxylin and eosin (H&E) staining.²⁵ The histological grading was determined based on tubular formation, nuclear pleomorphism, and mitotic index.²⁶

3. Patient follow-up

All CMNs cases included in this study underwent a structured follow-up protocol to assess clinical outcomes and survival. The total follow-up period extended from the day of mastectomy (D0) to the last recorded contact, with a maximum follow-up of 30 months. Three clinical and laboratory evaluations were performed at predefined time points, with laboratory reassessments conducted on the day of mastectomy (D0), at 21 days post-surgery (D1), and three months post-surgery (D2). These evaluations included physical examinations, hematological and biochemical analyses (renal and hepatic evaluation), and measurement of CA 15-3 levels. Beyond the three-month postoperative period, long-term follow-up was conducted through telephone interviews with owners, focusing on clinical status, potential recurrence of neoplastic lesions, and survival status. For all patients, the date of last contact was recorded. In cases of confirmed death, both the date and the presumed cause of death were documented in

4. Serum biomarkers CA 15-3

Peripheral venous blood samples were obtained from all subjects of the 5 groups with CMNs during the preoperative stage (D0) and routine checkups (D1 and D2) and on the day of the clinical evaluation for the control group. After collection and clot retraction, the samples were centrifuged at 1,450g for 5 min. Serum tests were performed according to the manufacturer's protocol. All assays were performed in duplicate, and a positive and negative control (supplied with the kit) with a known concentration range were included. CA 15-3 (IU/mL) levels were dosed using a commercial kit (BR-MA, Simens®), with an enzyme-linked fluorescent testing method (ELFA) using a two-step conjugation technique that conducts automated dosing with the IMMULITE 1000 Immunoassay System (Siemens®) with a measurement range of 2 to 400 IU/mL. Up to the present date, there were no determined reference intervals for serum levels of CA 15-3 in healthy female dogs.

5. Immunohistochemistry (IHC)

IHC analysis was performed on 3 µm thick sections obtained from formalin-fixed, paraffin-embedded CMNs samples. For antigen retrieval, slides were incubated in 0.01 M sodium citrate buffer (pH 6.0) at 99.8 °C for 10 minutes. Endogenous peroxidase activity was blocked using Peroxidase Block

(Novolink Max Polymer Detection System, Leica Biosystems®). The sections were incubated overnight at 4 °C with either monoclonal mouse anti-Ki-67 antibody (clone MIB-1, Dako®, M7240; 1:50) or rabbit monoclonal anti-COX-2 antibody (clone SP21, ThermoFisher Scientific®; 1:100). Detection was carried out with DAB chromogen, followed by counterstaining with Mayer's hematoxylin.

Ki-67 index was determined by manual counting of 1,000 cells in hotspot areas under 400× magnification. The proliferative index was expressed as the percentage of positively stained nuclei¹⁴.

COX-2 expression was evaluated semi-quantitatively, considering both the proportion of stained cells and the intensity of cytoplasmic staining in five high-power fields (400×). Distribution scores were defined as follows: 0 (absent), 1 (≤10%), 2 (11–30%), 3 (31–60%), and 4 (>60%). Staining intensity was graded from 0 (negative) to 3 (strong). The final score, obtained by multiplying distribution and intensity scores (range 0–12), was categorized as low (0–5) or high (6–12), following the criteria described by Lavalle *et al.* (2009) and adopted by Araújo *et al.* (2016).

6. Statistical analysis

Statistical analyses were performed using R (v.4.3.0) and Python (v.3.9), considering $p < 0.05$ as the significance threshold. Data distribution and variance homogeneity were evaluated using Shapiro-Wilk, Kolmogorov-Smirnov, and Levene's tests, and appropriate statistical methods were applied accordingly. Differences in CA 15-3 levels (D0, D1, and D2), Ki-67 and COX-2 expression between clinical stages were assessed using the Mann-Whitney U test, with Bonferroni correction for multiple comparisons. Longitudinal variations in CA 15-3 levels were analyzed using the Wilcoxon signed-rank test, and Spearman's correlation was used to evaluate associations with CMNs-related variables. Kaplan-Meier survival curves were generated for clinical staging, CA 15-3 levels, Ki-67 and COX-2 expressions, with group comparisons performed using the Log-Rank test. Additionally, Receiver Operating Characteristic (ROC) curves were used to determine the optimal CA 15-3, Ki-67 and COX-2 cutoff for distinguishing malignant from benign CMNs, while Principal Component Analysis (PCA) was applied to explore clustering patterns.

RESULTS

1. Characterization of the study population

In the control group, there was an average age of 2.33 ± 0.65 years and an average weight of 18.83 ± 3.89 kg. In the 54 CMNs, the average age of the age was 9.85 ± 3.43 years, with a predominance of elderly animals. Their weight ranged from 2.4 to 41 kg, with an average of 10.12 ± 7.35 kg. Regarding breed distribution, 51.85% of the dogs were mixed breed, followed by Pinscher (14.81%), Poodle (9.26%), and Yorkshire Terrier (5.56%). Most of the animals were intact (83.33%), while 16.67% were spayed.

Among the cases of CMNs, 16.67% were classified as benign, while 83.33% (45/54) were malignant (Figure 1). Stage II was the most prevalent, representing 24.07% of the cases, followed by stages I (20.37%), III (20.37%), and IV (14.81%).

Regarding the histopathological grade (Table 1), the results indicated a statistically significant difference between the clinical staging groups ($p = 0.0102$), suggesting that CMNs in more advanced stages present higher histological grades. The post-hoc test revealed that this difference was more evident between the extreme stages of CMNs progression, with significant differences between stages I and IV ($p = 0.0076$) and stages II and IV ($p = 0.0426$).

2. Serum CA 15-3 levels in the control group

Notably, serum CA 15-3 levels in the control group were below the detection threshold of the assay, preventing quantitative measurement. This result is consistent with our previous findings

(Pineiro *et al.*, 2023), where CA 15-3 concentrations in healthy dogs were either undetectable or at the lower limit of detection. For statistical analyses, these values were assigned to be the lowest detectable unit of the assay. Due to this, statistically significant differences were observed between the control group and all other groups according to clinical staging.

3. Levels of biomarkers by clinical staging in the CMN group

Serum CA 15-3 levels at D0 showed a significant association with clinical staging, with higher concentrations observed in more aggressive CMNs (stage III and IV) (Table 2). CA 15-3 levels were significantly higher in all malignant groups (I-IV) compared to the benign group ($p < 0.05$). However, no statistical difference was observed between stages II and III or stages III and IV ($p > 0.05$). Similar trends were found in CA 15-3 levels at D1 (21 days post-mastectomy) and D2 (3 months post-surgery), suggesting a persistent increase in more aggressive CMNs.

4. Temporal changes in CA 15-3 levels in CMN group

The longitudinal analysis of CA 15-3 levels over time (D0 → D1 → D2) showed that in stage I CMNs, CA 15-3 levels significantly decreased over time ($p = 0.0146$). In contrast, in benign group and stages II-IV, no statistically significant reduction was observed, indicating that CA 15-3 remained elevated in more aggressive neoplasms for three months.

5. IHC expression and clinical staging in the CMN group

Ki-67 and COX-2 IHC analysis did not show significant differences between clinical subgroups ($p > 0.05$ for all comparisons) (Table 2).

6. Correlations Between CA 15-3 and CMNs Characteristics

Spearman's correlation analysis demonstrated that CA 15-3 (D0) was strongly correlated with CMNs stage ($s = 0.59$, $p < 0.0001$), grade ($s = 0.64$, $p < 0.0001$), and the number of nodules ($s = 0.42$, $p = 0.0014$). However, no correlation was found between CA 15-3 and Ki-67 ($s = 0.13$, $p = 0.366$). Similarly, necrosis and ulceration did not show significant correlations with CA 15-3 levels. Conversely, Ki-67 was only significantly correlated with CMNs grade ($s = 0.41$, $p = 0.005$), but not with staging, size, or CA 15-3 levels ($p > 0.05$ for all comparisons). COX-2 expression did not show significant correlations with any of the analyzed variables ($p > 0.05$ for all comparisons).

7. Diagnostic accuracy of CA 15-3, Ki-67 and COX-2

Receiver operating characteristic (ROC) analysis identified an optimal CA 15-3 cutoff value of 3.01 IU/mL, with high sensitivity (93.0%) and specificity (90.9%) for distinguishing malignant from benign CMNs (AUC = 0.92). Ki-67 showed moderate discriminatory power, with an optimal cutoff of 15.8%, sensitivity of 56.1%, and specificity of 77.8% (AUC = 0.63) (Figure 2). ROC curve for COX-2, demonstrated poor discriminative performance (AUC = 0.44) with an optimal cutoff of 6.00 ng/mL, yielding 66.7% sensitivity and 33.3% specificity.

8. Survival analysis findings

8.1 Clinical staging

The Kaplan-Meier survival analysis stratified by clinical staging (Benign, I, II, III, IV) showed no statistically significant differences in survival among the groups ($p = 0.56$) (Figure 3). However, a pattern was observed in which patients with more advanced clinical stages (particularly stage IV) exhibited a steeper decline in survival probability compared to lower-stage groups, suggesting a potential trend toward reduced survival in these cases.

8.2 CA 15-3 at different time points

Survival analysis based on CA 15-3 tertile at different time points (D0, D1, and D2) did not reveal statistically significant differences among the low, medium, and high groups (Figure 4). At D0 (mastectomy day), no significant associations were found ($p = 0.12$ for low vs. medium, $p = 1.00$ for low vs. high, and $p = 0.06$ for medium vs. high), although a more pronounced decline in survival curves was observed in patients with higher CA 15-3 values, particularly in the high category. A similar trend persisted at D1 (21 days post-surgery), with no significant differences ($p = 0.71$ for low vs. medium, $p = 1.00$ for low vs. high, and $p = 0.46$ for medium vs. high), yet patients with elevated CA 15-3 levels showed a visibly steeper decrease in survival probability. By D2 (3 months post-surgery), survival differences remained statistically insignificant ($p = 0.44$ for low vs. medium, $p = 0.73$ for low vs. high, and $p = 0.14$ for medium vs. high); however, patients with higher CA 15-3 levels continued to exhibit a more rapid decline in cumulative survival over time.

8.3 IHC expression and proliferative index

Kaplan-Meier analysis of Ki-67 and COX-2 categorized into low, medium, and high groups did not demonstrate any statistically significant impact on survival. However, a tendency toward lower survival probability was noted in patients with higher Ki-67 levels, as evidenced by the steeper descent in survival curves in the High category (Figure 5).

9. Principal component analysis (PCA)

PCA incorporating CMNs grade, number of nodules, ulceration, necrosis, diameter, clinical staging, CA 15-3 levels, Ki-67 and COX-2 score revealed that the first two principal components explained 58.26% of the total variance. PC1 accounted for 40.30% of the variance and was predominantly influenced by clinical staging, CMNs grade, CA 15-3 levels, number of nodules, and diameter. PC2 explained 17.96% of the variance and was mainly associated with Ki-67 expression, ulceration, COX-2 score, and necrosis. Dimensionality reduction enabled visualization of CMNs grouping according to staging, with advanced stage (III and IV) distributed along the higher PC1 axis.

A multivariable PCA biplot with biomarker expression tertiles, histologic grade, and clinical staging demonstrated overlapping but distinguishable clusters. CMNs with higher CA 15-3 concentrations, COX-2 scores, and Ki-67 expression were more frequently located along the right side of the PC1 axis, close to high-grade and advanced-stages (Figure 6).

DISCUSSION

This study comprehensively evaluated the prognostic relevance of serum CA 15-3 and IHC expression of Ki-67 and COX-2 in CMNs, integrating survival analysis and multivariate statistical approaches. The findings reinforce the potential of these biomarkers to stratify disease severity and predict outcomes in CMNs.

Elevated serum CA 15-3 concentrations were significantly associated with advanced clinical stage, larger diameter, and higher histological grade, corroborating previous findings that linked this biomarker to CMNs burden and metastatic progression.⁸⁻¹⁰

Higher serum concentrations of CA 15-3 were more frequently observed in dogs with malignant CMNs, particularly when levels exceeded the ROC-derived threshold of 3.01 IU/mL, suggesting a potential diagnostic relevance. Additionally, Kaplan–Meier survival curves visually indicated that these patients tended to exhibit lower overall survival rates compared to those with lower CA 15-3 levels. However, statistical significance was not reached, possibly due to sample size constraints or the intrinsic variability of circulating biomarkers.

Although CA 15-3 has been proposed as a non-invasive marker for CMNs burden and recurrence risk, its prognostic utility remains debated.²⁷ The lack of significant survival differences in the present study reinforces the need for a multimodal approach that integrates serum and tissue biomarkers for more precise prognostication. Furthermore, the decline in CA 15-3 levels three months post-surgery suggests that serial measurements may offer additional insights into disease progression and treatment response, warranting further investigation.

Regarding Ki-67, the increased proliferative index was positively correlated with higher histological grades and increased aggressiveness, including necrosis and ulceration, supporting its role as a proliferation marker.¹¹ These findings are in line with previous literature.^{13,14,28}

Despite its established association with progression, Ki-67 alone may not be sufficient for survival prediction due to its heterogeneous expression within CMNs and potential sampling bias. Notably, our prior work demonstrated hormonal modulation of Ki-67 expression during the estrous cycle in normal canine mammary glands, highlighting its physiological and neoplastic significance in canine mammary glands¹⁶. Additionally, as a tissue-based biomarker, Ki-67 is inherently limited in real-time disease monitoring compared to serum biomarkers such as CA 15-3. These findings underscore the importance of integrating Ki-67 with additional prognostic markers to improve risk stratification and guide clinical decision-making.

Although prior studies have suggested that COX-2 expression correlates with neoplasms aggressiveness in CMNs, our findings did not reveal statistically significant associations between COX-2 immunoscores and key pathological or clinical variables, including histological grade, size, ulceration, necrosis, or clinical staging. Moreover, its predictive capacity for malignancy was limited, as indicated by a low AUC value (0.44) and the absence of survival differences across expression groups. These results diverge from those reported in earlier studies, possibly due to differences in CMNs subtype distribution, and antibody clones.¹⁸⁻²²

Nonetheless, principal component analysis highlighted the contribution of COX-2 to the second component, alongside ulceration, necrosis, and Ki-67, indicating that while COX-2 may not independently stratify prognostic risk, when used with other markers can be useful in the characterization of CMNs aggressiveness. This supports the notion that COX-2 prognostic value may be context-dependent and better understood within multiparametric frameworks. Given its role in inflammation and therapeutic relevance as a target for NSAIDs, COX-2 remains a candidate biomarker in CMNs, though its utility may be optimized through integrative models and standardized evaluation protocols.²⁹⁻³¹

Despite the individual prognostic value of CA 15-3, Ki-67, and COX-2, few veterinary studies have integrated these markers into a unified survival analysis model. Our results suggest that combining serum and tissue biomarkers improves risk stratification and may better inform treatment decisions. This integrative biomarker approach reflects current trends, where combining serum and tissue markers enhances diagnostic and prognostic accuracy.^{4,27}

Nevertheless, some limitations must be acknowledged. The sample size, though sufficient for exploratory analysis, restricts the generalizability of our findings. Additionally, while CA 15-3 exhibited promising trends, its clinical applicability in canine patients requires further validation through larger, longitudinal studies with standardized cutoff values. Future research should also explore the potential of additional serum biomarkers to improve prognostic accuracy and facilitate early recurrence detection.

This study demonstrates that serum CA 15-3 levels, Ki-67 proliferative index, and COX-2 expression are relevant biomarkers associated with clinical and pathological features of CMNs. Elevated CA 15-3 concentrations, particularly on day 0 (D0), were significantly correlated with advanced clinical stages. Ki-67 expression was positively associated with grade, reinforcing its

prognostic value. Despite COX-2 showing a weaker correlation with survival, its IHC scoring exhibited moderate alignment with CMNs progression parameters in principal component analysis.

The combination of serum and tissue biomarkers may improve the stratification of prognosis and guide therapeutic decision-making in veterinary oncology. These findings support the growing role of biomarker panels in the clinical management of CMNs and emphasize their potential translational relevance to human breast cancer.

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Table 1. Clinical and histopathological indices in female dogs with CMNs, according to study groups.

	B	I	II	III	IV
Most frequent breed	Pinscher (33.33%)	SRD (54.5%)	SRD (53.8%)	SRD (58.3%)	SRD (62.5%)
Age (years)	8.4 ± 3.9	11.5 ± 4.3	8.8 ± 3.0	9.3 ± 2.6	11.4 ± 2.3
Weight (kg)	9.4 ± 9.7	7.7 ± 4.1	9.2 ± 5.3	14.3 ± 10.5	9.4 ± 3.9
Reproductive status	Intact (100%)	Intact (63.6%)	Intact (100%)	Intact (83.3%)	Intact (75.0%)
Number of nodules	1.2 ± 0.4	2.3 ± 1.1	1.8 ± 1.3	2.3 ± 1.0	3.1 ± 2.3
Most affected mammary gland	M5 (55.5%)	M3 (36.3%)	M5 (38.5%)	M5 (41.7%)	M5 (50.0%)
Nodule diameter (cm)	1.4 ± 1.5	1.3 ± 0.7	2.7 ± 0.9	5.1 ± 3.2	7.5 ± 5.3
Most frequent diagnosis	Complex adenoma (33.33%)	Complex carcinoma (54.5%)	Mixed tumor carcinoma (38.4%)	Complex carcinoma (25.0%)	Comedocarcinoma (25.0%)
Grade	†	1.2 ± 0.4 ^a	1.4 ± 0.5 ^a	1.8 ± 0.9 ^b	2.2 ± 0.7 ^b

The table presents the distribution of clinical and histopathological variables in female dogs with CMNs, stratified into benign neoplasms (B) and carcinomas according to clinical staging (I, II, III, and IV). Inguinal mammary gland (M5) and cranial abdominal mammary gland (M3). Numerical values are presented as mean ± standard deviation, and relative frequencies are expressed as percentages (%). † Not applicable to this subgroup of the population. Superscript letters within the same line indicate statistically significant differences.

Table 2. Biomarker values for CA 15-3 at different time points (D0, D1, and D2), Ki-67 and COX-2 in female dogs with CMNs, according to study groups.

Staging	CA 15-3 (D0) (IU/mL)	CA 15-3 (D1) (IU/mL)	CA 15-3 (D2) (IU/mL)	Ki-67 (%)	COX-2 (score)
B	2.32 ± 0.46 ^a	1.99 ± 0.37 ^a	1.62 ± 0.30 ^a	12.43 ± 11.88 ^a	1.78 ± 1.56 ^a
I	7.71 ± 3.15 ^b	6.70 ± 2.76 ^b	5.41 ± 2.20 ^b	14.93 ± 12.78 ^a	2.11 ± 1.83 ^a
II	5.80 ± 2.31 ^{bc}	5.16 ± 2.14 ^{bc}	4.23 ± 1.78 ^{bc}	24.38 ± 23.05 ^a	2.18 ± 2.23 ^a
III	9.71 ± 5.11 ^c	8.38 ± 4.58 ^c	6.58 ± 3.74 ^{bc}	21.84 ± 15.67 ^a	0.60 ± 0.97 ^a
IV	11.03 ± 5.16 ^c	9.93 ± 4.79 ^c	8.32 ± 4.30 ^{bc}	26.75 ± 19.52 ^a	1.50 ± 2.14 ^a

The table presents the mean ± standard deviation values of the biomarkers CA 15-3 (IU/mL), at different time points (D0, D1, and D2), Ki-67 (%) and COX-2 score according to clinical staging (B, I, II, III, and IV) in CMNs. Superscript letters within the same column indicate statistically significant differences.

Figure 1. Histological subtypes of canine mammary neoplasms (H&E) and immunohistochemical expression of Ki-67 and COX-2 observed in the study. (A) Complex adenoma (10× objective); (B) Complex carcinoma (10× objective); (C) Carcinoma in mixed tumor (10× objective); (D) Comedocarcinoma (4× objective); all stained with H&E. (E) Fibroadenoma with low Ki-67

expression (20× objective); (F) Ductal carcinoma with high Ki-67 expression (20× objective); (G) Micropapillary carcinoma with low COX-2 expression (40× objective); (H) Carcinoma in mixed figure with high COX-2 expression (40× objective); all detections was carried out with DAB chromogen, followed by counterstaining with Mayer's hematoxylin. Scale bars are indicated in each panel.

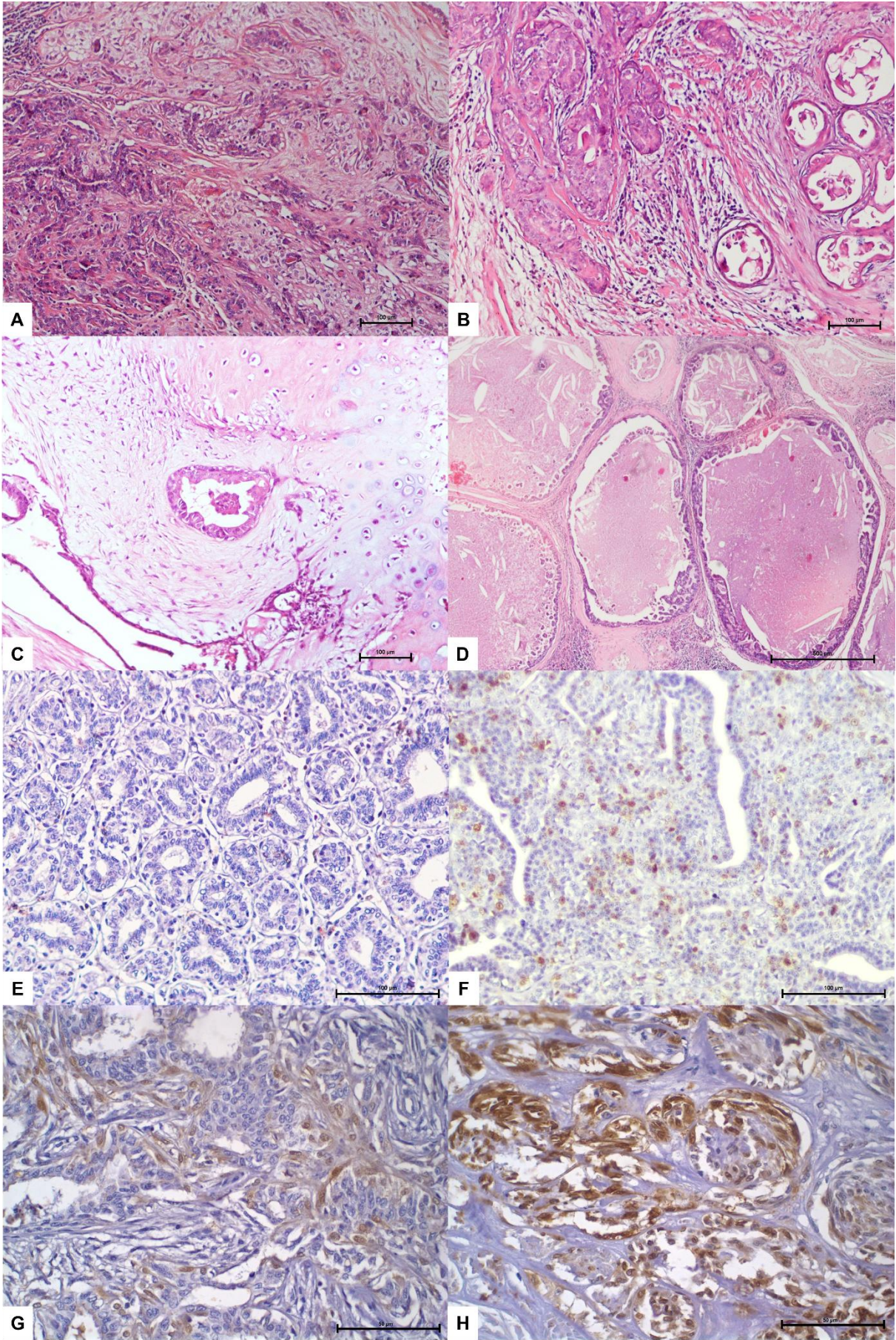


Figure 2. Receiver operating characteristic (ROC) curve analysis for CA 15-3, Ki-67, and COX-2 in distinguishing malignant from benign canine mammary neoplasms (CMNs). The upper left panel (yellow) displays the ROC curve for CA 15-3; the upper right panel (green) shows the ROC curve for Ki-67; and the lower panel (blue) presents the ROC curve for COX-2.

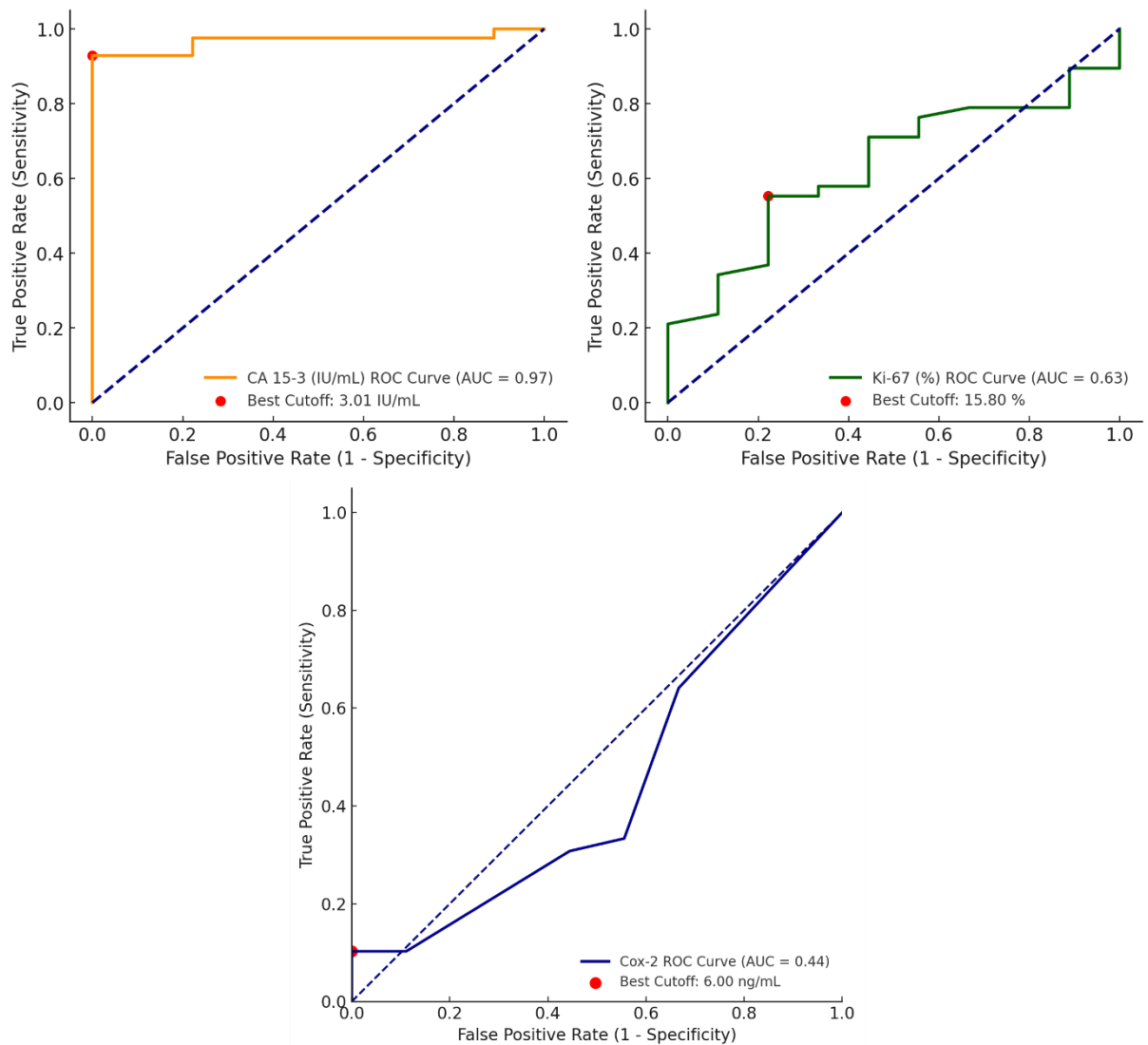


Figure 3. Kaplan-Meier survival analysis of CMNs across stage groups. This Kaplan-Meier survival curve illustrates the cumulative survival probability over time (in months) for CMNs stratified by clinical staging (benign, I, II, III, and IV). Each line represents a different staging group, with distinct colors and markers differentiating them.

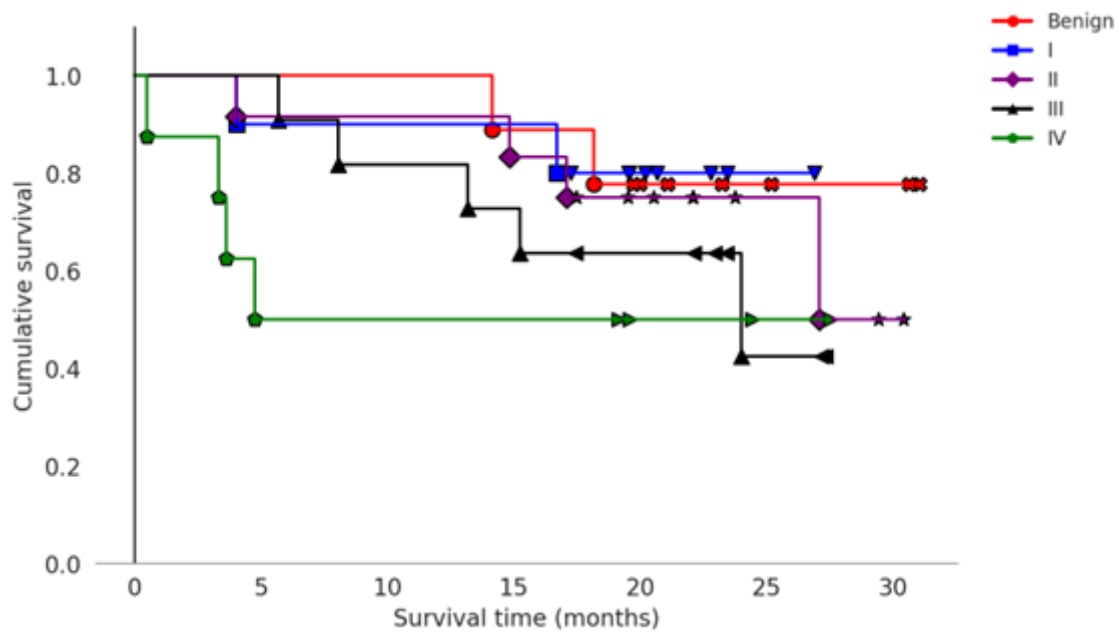


Figure 4. Kaplan–Meier survival curves stratified by serum CA 15-3 levels at three time points. (A) D0: day of mastectomy (top left); (B) D1: 21 days post-surgery (top right); (C) D2: 3 months post-surgery (bottom center). Survival probability progressively decreases over time, with distinct patterns observed among dogs with low (blue), medium (yellow), and high (red) CA 15-3 concentrations.

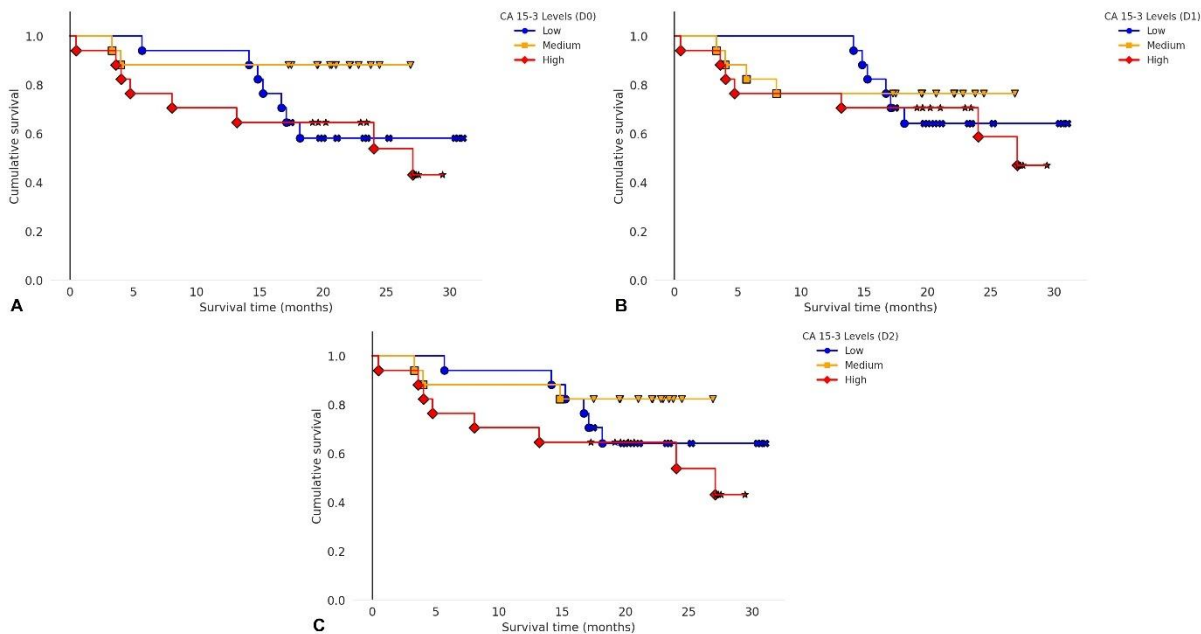


Figure 5. Kaplan–Meier survival curves stratified by Ki-67 and COX-2 expression levels in canine mammary neoplasms (CMNs). (A) Ki-67 proliferative index; (B) COX-2 score. Survival probability progressively decreases over time, with distinct patterns observed among dogs with low (blue), medium (yellow), and high (red) Ki-67 or COX-2 expression levels.

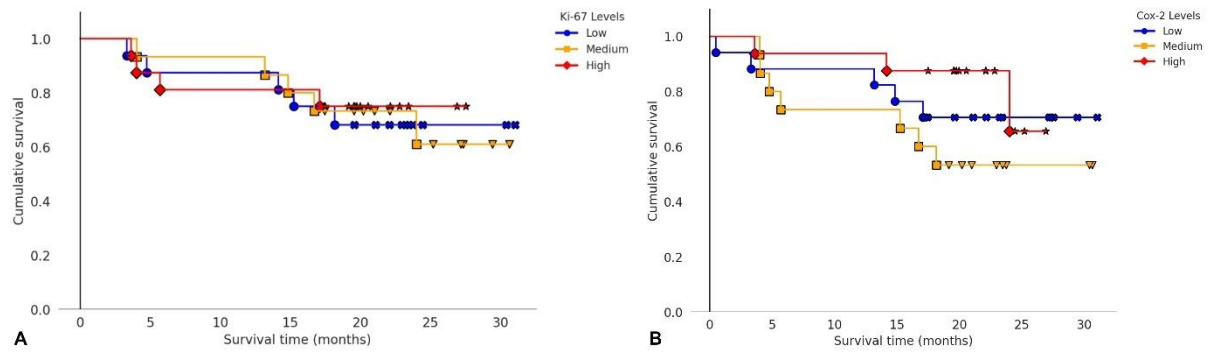
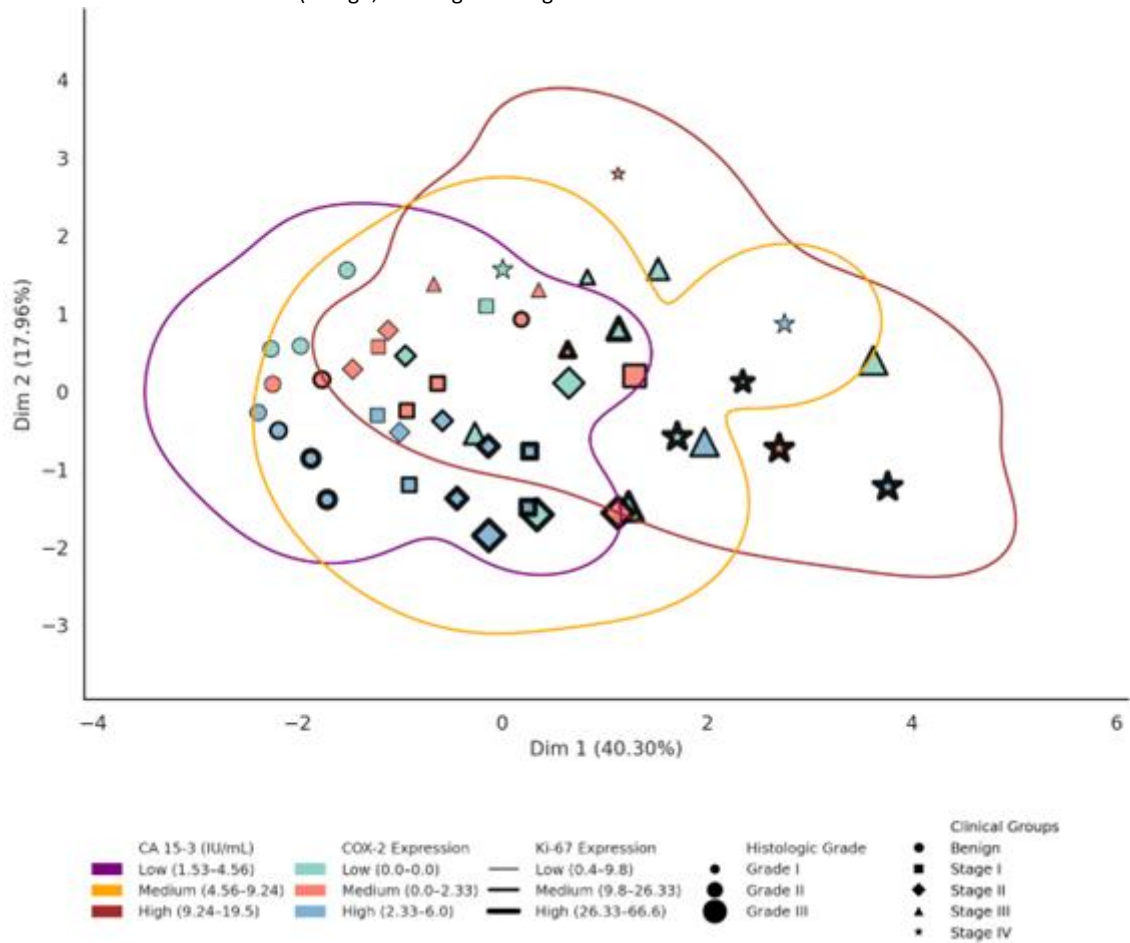


Figure 6. Multidimensional distribution of clinical and pathological parameters across CA 15-3 terciles. CMNs are grouped according to serum CA 15-3 concentration terciles, represented by colored contours: low (≤ 4.56), medium (4.56–9.24), and high (≥ 9.24). Each point corresponds to a single patient and simultaneously encodes several dimensions of CMNs behavior: the color reflects COX-2 expression level (low to high), the thickness of the black outline represents Ki-67 expression (thicker borders indicate higher proliferation), the size of the point corresponds to histologic grade (from grade I to III), and the shape indicates clinical classification (benign, or malignant stages I–IV).



8 CONCLUSÃO

A presente tese integrou dois eixos investigativos complementares, com o intuito de aprofundar a compreensão da biologia da glândula mamária canina em contextos fisiológicos e patológicos, alinhando-se aos objetivos delineados. No primeiro eixo, a análise imuno-histoquímica dos índices proliferativo (Ki-67) e apoptótico (Caspase-3) revelou que o ciclo estral exerce influência significativa sobre a dinâmica celular da mama normal, especialmente durante o início do diestro, quando se observou um aumento expressivo da atividade proliferativa. Esse aumento mostrou forte correlação com os níveis séricos de progesterona, confirmando que o contexto hormonal é determinante na regulação da proliferação e apoptose nos diferentes compartimentos histológicos da glândula mamária. Esses achados ressaltam a importância de considerar o estágio do ciclo estral e os níveis hormonais na interpretação de marcadores celulares, tanto em estudos fisiológicos quanto em análises neoplásicas.

No segundo eixo, a avaliação de biomarcadores prognósticos em neoplasias mamárias caninas demonstrou que o antígeno CA 15-3 apresenta elevado potencial discriminativo na diferenciação entre neoplasias benignas e malignas, com correlações significativas com o estadiamento clínico e o grau histológico. A expressão tecidual de Ki-67 reforçou sua utilidade como marcador de agressividade tumoral, enquanto a COX-2, embora de desempenho limitado isoladamente, mostrou relevância contextual quando combinada em modelos multiparamétricos. A integração entre marcadores séricos e teciduais demonstrou maior robustez para estratificação prognóstica e interpretação de sobrevida, evidenciando a importância de uma abordagem integrada que considere tanto os dados fisiológicos da glândula normal quanto os parâmetros clínico-patológicos dos tumores.

9 PERSPECTIVAS

O trabalho desenvolvido nesta tese reforça uma noção central: a glândula mamária canina é um tecido altamente dinâmico, regulado por mecanismos hormonais que impactam diretamente os parâmetros utilizados na oncologia veterinária como critérios prognósticos. Desconsiderar essa plasticidade fisiológica pode resultar em interpretações enviesadas da agressividade tumoral, especialmente em casos borderline. Nesse sentido, os achados sustentam a necessidade de abordagens integrativas e contextualizadas, nas quais biomarcadores moleculares e hormonais sejam analisados em conjunto. Do ponto de vista aplicado, esta tese

aponta para a utilidade do CA 15-3 como ferramenta não invasiva de monitoramento e estratificação prognóstica, bem como para o potencial da associação entre Ki-67 e COX-2 na construção de perfis de risco mais precisos. Além disso, os dados obtidos sobre a glândula normal fornecem parâmetros de referência inéditos para protocolos experimentais, diagnósticos diferenciais e abordagens terapêuticas mais alinhadas à fisiologia da paciente.

Apesar dos avanços, o estudo enfrentou limitações relevantes, sobretudo relacionadas ao acompanhamento clínico das pacientes e à dificuldade em estabelecer, de forma inequívoca, a causa da morte. Tais desafios reforçam a importância de dar continuidade a este esforço, por meio da padronização de coleções de *follow-up* clínico e necroscópico, de modo a consolidar bases de dados mais robustas e comparáveis. A continuidade desse trabalho visa não apenas superar fragilidades metodológicas, mas também contribuir para a consolidação de protocolos que ampliem a precisão diagnóstica, o valor prognóstico e o impacto translacional das pesquisas em oncologia veterinária.

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ANEXO A – CERTIFICADO DE APROVAÇÃO DO COMITÉ DE ÉTICA



Comissão de Ética para o Uso de Animais
Av. Dr. Silas Munguba, 1700 – Itaperi
CEP 60740-903 – fone 3101-9890
ceua.uece@uece.br – www.uece.br/ceua



CERTIFICADO

Certificamos que o Projeto intitulado “Relação do biomarcadores CA15-3 e painel de miRNA séricos com a avaliação imunohistoquímica tecidual e sobrevida global de cadelas com carcinomas mamários”, registrado sob o número 11690648/2021, tendo como pesquisador principal Lúcia Daniel Machado da Silva, está de acordo com os Princípios Éticos de Experimentação Animal adotados pela Comissão de Ética para o Uso de Animais da Universidade Estadual do Ceará (CEUA – UECE). Este certificado expira-se em 31 de Dezembro de 2025.

CERTIFICATE

We hereby certify that the Project entitled “Relationship of CA15-3 biomarkers and serum miRNA panel with tissue immunohistochemical evaluation and overall survival of bitches with breast carcinomas” registered with the protocol 11690648/2021, under the supervision of Lúcia Daniel Machado da Silva, is in agreement with Ethical Principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation of Ceará State University (CEUA – UECE). This certificate will expire on December 31st, 2025.

RESUMO

Vigência do projeto	Início	10/01/2022	Fim	31/12/2025
Espécie/Linhagem	Canis lupus familiaris SRD			
Número de animais		Peso	Não definido	Idade >06 anos
Sexo	80	Feminino		Masculino
Origem	Hospital Veterinário Prof Sylvio Barbosa Cardoso - HVSBC			
Metodologia	X	Adequada		Não adequada
Cronograma	X	Adequado		Ausente/ Não adequado
Ofício de encaminhamento	X	Presente		Ausente/ Não adequado
Orçamento	X	Adequado		Ausente/ Não adequado
Anuência de Laboratórios Parceiros/Proprietários/Outros	X	Presente		Ausente/ Não adequado
Financiamento	Órgão de fomento		Recursos de Pesquisa e Parcerias	
	Edital ou N. processo		-	

Fortaleza, 03 de Janeiro de 2022.

Vania Marilande Ceccatto
Presidente CEUA-UECE

ANEXO B – COMPROVANTE DE SUBMISSÃO DO CAPÍTULO II*For consideration in Veterinary and Comparative Oncology*

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Veterinary and Comparative Oncology

Original Article

Serum CA 15-3 and immunohistochemical Ki-67 and COX-2 expression in canine mammary neoplasms: biomarkers for prognosis and survival prediction

Submission ID 3e010cfb-997e-474a-9a70-7cddcbc5abed

Manuscript ID draft-3e010cfb-997e-474a-9a70-7cddcbc5abed

Submission Version Initial Submission

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