



UNIVERSIDADE ESTADUAL PAULISTA
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Câmpus de São José do Rio Preto

Isabela Fernanda Spinelli Perossi

**Validação da variante c.3140A>G e de suas potenciais proteínas
alvo relacionadas a via PIK3CA/AKT/mTOR em neoplasias
mamárias de cadelas**

São José do Rio Preto
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“Algumas pessoas passam por sua vida, outros a acompanham até que não lhes seja mais possível, outros estão mais perto do que parecem.”

A menina que roubava livros (Markus Zusak, 1975, p.180)

RESUMO

O câncer de mama (CM) é um relevante problema de saúde pública, pois representa a segunda principal causa de morte relacionada ao câncer em mulheres em todo o mundo. Os cães são utilizados como modelo para estudos desses tumores, pois além da grande ocorrência, possuem semelhanças biológicas e fisiopatológicas. A via PIK3CA/AKT/mTOR está fortemente desregulada no câncer de mama e desempenha papel central na homeostase celular. A literatura recente retrata que todas as mutações somáticas identificadas a partir do gene PIK3CA alteraram as sequências de aminoácidos, dentre eles a variante c.3140A> G. Esse gene participa da regulação de uma ampla gama de atividades celulares relevantes englobando várias proteínas importantes. Na medicina veterinária o estudo desta via é incipiente. O objetivo deste trabalho foi analisar a variante c.3140A>G do gene PIK3CA e a expressão das proteínas alvo PIK3CA, PTEN, HIF, VHL, ZEB1, ZEB 2, Caspase-3 e PARP1 como marcadores prognósticos em um estudo prospectivo, verificando a expressão gênica dessa variante e, em um estudo retrospectivo, avaliando a expressão proteica e gênica de alvos moleculares à jusante da via correlacionando com o prognóstico nas cadelas. No estudo retrospectivo, o DNA de 24 fragmentos de tumores mamários caninos e 20 fragmentos normais de mama de cadelas foi extraído e a mutação verificada usando TaqMan® Mutation Detection Assay. A imunohistoquímica foi realizada com o kit Reveal HRP Conjugate e sua análise pelo método Histoscore. Duas pacientes (8,3%) apresentaram a mutação c.3140A> G, sendo que uma paciente apresentou metástase pulmonar e morreu em 30 dias após o diagnóstico e a outra paciente permanece viva. Os dados de imunohistoquímica revelaram que a expressão das proteínas PIK3CA, HIF, ZEB2 e PARP1 foi maior na paciente que veio a óbito em comparação com a que permanece viva ($p < 0,05$). No estudo prospectivo, para a análise proteica as amostras foram provenientes de 58 cadelas com neoplasia mamária, previamente identificadas por análise histopatológica. A imunohistoquímica foi realizada com o kit Reveal HRP Conjugate e sua análise pelo método Histoscore. Para a análise genica as amostras são provenientes de 13 pacientes caninas acometidas por neoplasia mamária primária, o DNA foi extraído segundo o método de Sambrook & Russel e realizada a análise para expressão quantitativa utilizando o sistema Step One Plus. Através da imunohistoquímica foi observado que a positividade de PIK3CA foi significativamente associada a linfonodo regional

acometido, à metástase a distância, às pacientes com fenótipos HER2+, Triplo Negativo e Luminal B e também à menor sobrevida. Por meio da expressão gênica observamos, entre as pacientes vivas e com óbito, expressão gênica mais elevada de ZEB2 e PARP1 comparado aqueles sem metástase, o que não foi verdadeiro para as expressões de PIK3CA e HIF1 α . Em conclusão, os dados observados neste trabalho são promissores no estudo de novos marcadores moleculares de prognóstico adequados para o desenvolvimento de novas terapias, ainda assim mais pesquisas destinadas a esclarecer e validar as relações entre a via PI3K/AKT/mTOR.

Palavras-chave: Neoplasia mamária. Via PI3K/AKT/mTOR. Expressão proteica. Expressão gênica. Prognóstico. Metástase.

ABSTRACT

Breast cancer (BC) is a relevant public health problem, as it represents the second leading cause of cancer-related death in women worldwide. Dogs are used as a model for studies of these tumors, because in addition to their high occurrence, they have biological and pathophysiological similarities. The PIK3CA/AKT/mTOR pathway is strongly dysregulated in breast cancer and plays a central role in cellular homeostasis. Recent literature shows that all somatic mutations identified from the PIK3CA gene altered the amino acid sequences, including the variant c.3140A>G. This gene participates in the regulation of a wide range of relevant cellular activities encompassing several important proteins. In veterinary medicine, the study of this pathway is incipient. The objective of this work was to analyze the c.3140A>G variant of the PI3KCA gene and the expression of the target proteins PIK3CA, PTEN, HIF, VHL, ZEB1, ZEB 2, Caspase-3 and PARP1 as prognostic markers in a prospective study, verifying the gene expression of this variant and, in a retrospective study, evaluating the protein and gene expression of molecular targets downstream of the pathway correlating with the prognosis in bitches. In the retrospective study, DNA from 24 canine mammary tumor fragments and 20 canine normal mammary fragments was extracted and mutation verified using the TaqMan® Mutation Detection Assay. Immunohistochemistry was performed with the Reveal HRP Conjugate kit (Spring®) and its analysis was performed using the Histoscore method. Two patients (8.3%) had the c.3140A>G mutation, one patient had lung metastasis and died within 30 days of diagnosis and the other patient remains. Immunohistochemistry data revealed that the expression of PIK3CA, HIF, ZEB2 and PARP1 proteins was higher in the patient who died compared to the one who remained alive ($p < 0.05$). In the prospective study, for protein analysis, the samples came from 58 bitches with mammary neoplasia, previously identified by histopathological analysis, immunohistochemistry was performed with the Reveal HRP Conjugate kit (Spring®) and its analysis by the Histoscore method. For the genetic analysis, the samples come from 13 canine patients affected by primary mammary neoplasia, the DNA was extracted according to the method of Sambrook & Russell and the analysis for quantitative expression was performed using the Step One Plus system (Applied Biosystems®, Foster City, CA, USA). Through immunohistochemistry, PIK3CA positivity was significantly associated with affected regional lymph node, distant metastasis, patients with HER2+, Triple

Negative and Luminal B phenotypes and also the lowest survival in univariate analysis. Through gene expression, we observed higher gene expression of ZEB2 and PARP1 among patients who were alive and with death compared to those without metastasis, which was not true for the expressions of PIK3CA and HIF1 α . In conclusion, the data observed in this work are promising in the study of new molecular markers of prognosis suitable for the development of new therapies, yet more research is aimed at clarifying and validating the relationships between the PI3K/AKT/mTOR pathway.

Keywords: Breast cancer. PI3K/AKT/mTOR pathway. Protein expression. Gene expression. Prognosis. Metastasis.

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LISTA DE ABREVIATURAS E SIGLAS

AKT	do inglês <i>Protein kinase B</i>
AMPK	Proteína Quinase Ativada por Monofosfato de Adenosina
BCL-2	do inglês <i>B-cell lymphoma 2</i>
BCL-XL	do inglês <i>B-cell lymphoma-extra large</i>
BRCA1	do inglês <i>Breast Cancer 1</i>
BRCA2	do inglês <i>Breast Cancer 2</i>
CEUA	Comissão de Ética na Utilização de Animais
CMC	Carcinoma Mamário Canino
DAB	Diaminobenzidina
DNA	Ácido Desoxirribonucleico
EGF	do inglês <i>Epidermal Growth Factor</i>
EGFR	Receptor do Fator de Crescimento Epidérmico
EMT	Transição Epitelial-Mesenquimal
ER	Receptor de Estrógeno
FGFR	Receptor do Fator de Crescimento de Fibroblastos
FOXO1	do inglês <i>Forkhead Box Protein O1</i>
GPCRs	Receptores Acoplados à Proteína G
GSK-3	do inglês <i>Glycogen Synthase Kinase-3</i>
HER-2	Receptor do Fator de Crescimento Epidérmico Humano 2
HIF-1	Fator Induzível por Hipóxia
HR	Hazard ratio
HRP	do inglês <i>Horseradish Peroxidase</i>
HS	Histoscore
IGF-IR	Receptor do Fator de Crescimento Semelhante à Insulina I
IHC	Imunohistoquímica
IL-17	Interleucina-17
IL-1B	Interleucina-1 β
LR	Log rank
mTOR	do inglês <i>Mammalian Target of Rapamycin</i>
N-CAD	N-caderina
NF-κB	do inglês <i>Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells</i>

NGS	Sequenciamento de Próxima Geração
ONG	Organização Não Governamental
PARP	Poli (ADP-Ribose) Polimerase
PBS	do inglês <i>Phosphate-Buffered Saline</i>
PCR	do inglês <i>Polimerase Chain Reaction</i>
PDK1	Piruvato Desidrogenase Lipoamida Quinase-1
PI3K	Fosfatidilinositol-3 Quinase
PIK3CA	do inglês <i>Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha</i>
PIP2	Fosfatidilinositol-4,5-bifosfato
PIP3	Trifosfato de Fosfatidilinositol
PR	Receptor de Progesterona
PTEN	do inglês <i>Phosphatase with Tensin Homology Deleted in Chromosome 10</i>
RAS	do inglês <i>Rat Sarcoma Virus</i>
RNA	Ácido Ribonucleico
ROC	do inglês <i>Receiver Operating Characteristic</i>
RQ	Quantificação Relativa
RTKs	Receptores Tirosina Quinases
SLUG/SNAIL	do inglês <i>Zinc finger protein SNAI1</i>
TGF	do inglês <i>Transforming Growth Factor</i>
TMA	do inglês <i>Tissue Microarray</i>
TNF- α	do inglês <i>Tumor Necrosis Factor-A</i>
TWIST	do inglês <i>Twist-related protein 1</i>
VEGF	Fator de Crescimento Endotelial
VHL	Von Hippel-Lindau
VIM	Vimentina
ZEB1	do inglês <i>Zinc Finger E-Box Binding Homeobox 1</i>
ZEB2	do inglês <i>Zinc Finger E-Box Binding Homeobox 2</i>

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1 INTRODUÇÃO

1.1 Câncer de mama

Neoplasias mamárias afetam mulheres e cadelas em todo o mundo e, mesmo com o diagnóstico precoce e avanços na terapia, muitos casos são resistentes ao tratamento e a doença progride para metástase (AMORNSUPAK et al., 2017). O câncer de mama é a segunda principal causa de morte de mulheres em todo o mundo (HARBECK et al., 2019). Na veterinária, os tumores de glândulas mamárias são os mais frequentes nas cadelas, representando cerca de 50 a 70% de todas as neoplasias nessa espécie; outrossim estima-se o surgimento de um milhão de novos casos no mundo a cada ano, proporcionando alto risco de óbito em decorrência das metástases secundárias à essa neoplasia (CASSALI et al., 2014; NARDI et al., 2016).

Sabe-se que os receptores de estrógeno e progesterona estão intimamente envolvidos no mecanismo da formação de tumores mamários, uma vez que estimulam a proliferação do epitélio, ocorrendo aumento na possibilidade de ocorrência de mutações (PENÃ et al., 2014).

As neoplasias mamárias caninas compartilham várias semelhanças com o câncer de mama em mulheres, como a alta taxa de recorrência, de metástase e de mortalidade, além de características epidemiológicas, biológicas e genéticas (ABDELMEGEED e MOHAMMED, 2018; AMIRKHANI NAMAGERDI et al., 2020; GRAY et al., 2020). Entre estas, citam-se a faixa etária de acometimento, morfologia da lesão, efeito protetor da ovariosalpingohisterectomia, presença de receptores de estrógeno e progesterona no tecido tumoral e a hereditariedade, em alguns casos (QUEIROGA et al., 2014).

Além das semelhanças citadas, as neoplasias mamárias caninas compartilham características moleculares com o câncer de mama em mulheres (PASTOR et al., 2020; QUEIROGA et al., 2014), sendo assim, as neoplasias mamárias em cadelas são modelos apropriados e válidos ao estudo da biologia do câncer de mama em mulheres (ZUCCARI, et al., 2008).

Além dos fatores citados, atualmente o convívio estreito dos animais de companhia, faz com que seus tutores busquem por recursos que garantam qualidade de vida a seus animais e permitam o desenvolvimento de uma oncologia veterinária que objetiva, não apenas a compreensão das neoplasias como modelos

experimentais para humanos, mas também o bem estar desses (PORRELLO *et al*, 2006; BIONDI *et al*, 2014).

Como dito, o fator de risco mais significativo na ocorrência de tumor de mama em cadelas é a exposição hormonal, outros fatores incluem idade avançada, raça, obesidade aos 9 meses a 1 ano de idade e dieta com carne vermelha (BULMAN-FLEMING, 2020). A sobrevivência dos animais acometidos com a neoplasia é influenciada por vários fatores, como tipo e grau histopatológico, presença de metástases, estágio da doença e tratamento utilizado (SORENMO *et al.*, 2020).

Embora os avanços na oncologia sejam significativos, o câncer continua sendo um desafio na medicina humana e veterinária, por conseguinte, o diagnóstico precoce assume grande relevância, sendo obtido na rotina clínica, majoritariamente pelo exame histopatológico, no entanto, vem se tornando cada vez mais necessária a utilização de ferramentas mais avançadas para conseguir informações precisas sobre o tipo histológico e prognóstico da doença, como as análises de imuno-histoquímica e moleculares (CASSALI *et al.*, 2014; BORECKA *et al.*, 2020).

1.2 Métodos de diagnóstico e classificação

Fatores prognósticos clínicos e histológicos para tumores mamários foram previamente estudados e têm sido sugeridos como parâmetros importantes na determinação do possível desfecho e progressão da doença (SORENMO *et al.* 2013; CASSALI *et al.*, 2020).

Na área clínica as características mais importantes são o tamanho do tumor, o estado dos linfonodos e a presença de metástases à distância (YOSHIDA *et al.*, 2014). Esse estadiamento também conhecido como “TNM” é baseado, para a espécie canina, em OWEN *et al.* (1980) para obter informações a respeito do comportamento biológico da lesão, além do uso de marcadores moleculares que auxiliam no diagnóstico e também nos fatores prognósticos (ZUCCARI *et al.*, 2008). Este estadiamento clínico é relevante, uma vez que a detecção de metástase a distância pode alterar a conduta terapêutica (CASSALI, 2020).

A histopatologia é a principal ferramenta diagnóstica utilizada na medicina veterinária e deve ser realizada em todas as lesões mamárias, independentemente do tamanho. Diversos métodos de classificação histopatológica foram propostos para

os tumores mamários caninos e se baseiam na identificação morfológica das células permitindo um diagnóstico preciso em muitas neoplasias (CASSALI et al., 2014).

Na medicina humana a classificação histológica utilizada é a da Organização Mundial de Saúde (MISDORP et al., 1999). Atualmente, no Brasil, a classificação descrita por Cassali (2011) é comumente utilizada, já a classificação de Goldschmidt (2011) tem sido a mais adotada mundialmente e é descrita a seguir.

Tabela 1 – Classificação histológica proposta por Goldschmidt et al. (2011).

<p>1: Neoplasias Epiteliais Malignas.</p> <ul style="list-style-type: none"> • Carcinoma in situ. • Carcinoma simples. <ul style="list-style-type: none"> a. Tubular. b. Tubulopapilar. c. Cístico-papilar. d. Cribiforme. • Carcinoma micropapilar invasivo. • Carcinoma sólido. • Comedocarcinoma. • Carcinoma anaplásico. • Carcinoma decorrente de adenoma complexo/ tumor misto: é possível detectar áreas benignas na secção tecidual. • Carcinoma complexo: o componente epitelial é maligno e o mioepitelial é benigno. • Carcinoma e mioepitelioma maligno: os componentes epitelial e mioepitelial são malignos. • Carcinoma em tumor misto: o componente epitelial é maligno, o componente mesenquimal mioepitelial é benigno e o componente mesenquimal é cartilagem ou osso. • Carcinoma ductal: vertente maligna do adenoma ductal. • Carcinoma papilar intraductal: vertente maligna do adenoma papilar intraductal. 	<p>3: Neoplasias Mesenquimais Malignas – Sarcomas</p> <ul style="list-style-type: none"> • Osteossarcoma. • Condrossarcoma. • Fibrossarcoma. • Hemangiossarcoma. • Outros sarcomas.
<p>2: Neoplasias Epiteliais Malignas – Tipos Especiais</p> <ul style="list-style-type: none"> • Carcinoma de células escamosas. • Carcinoma adenoescamoso. • Carcinoma mucinoso. • Carcinoma secretório rico em lipídeos. • Carcinoma de células fusiformes. <ul style="list-style-type: none"> a. Mioepitelioma maligno. b. Carcinoma fusiforme de células escamosas. c. Carcinoma fusiforme. • Carcinoma inflamatório. 	<p>4: Carcinossarcoma – Tumor mamário misto maligno</p> <p>5: Neoplasias benignas</p> <ul style="list-style-type: none"> • Adenoma simples. • Adenoma papilar intraductal. • Adenoma ductal (adenoma basalóide). • Fibroadenoma. • Mioepitelioma. • Adenoma complexo • Tumor misto benigno
	<p>6: Hiperplasia/ Displasia</p> <ul style="list-style-type: none"> • Ectasia ductal. • Hiperplasia lobular (adenose). <ul style="list-style-type: none"> a. Regular b. Com atividade secretória (lactacional). c. Com fibrose d. Com atipia • Epiteliose. • Papilomatose. • Variação fibroadenomatosa. • Ginecomastia
	<p>7: Neoplasias do mamilo</p> <ul style="list-style-type: none"> • Adenoma. • Carcinoma. • Carcinoma com infiltração edidérmica.
	<p>8: Hiperplasia/ Displasia do mamilo</p> <ul style="list-style-type: none"> • Melanose da pele do mamilo.

Como observado na Tabela 1, de forma geral as alterações mamárias são classificadas em neoplasias epiteliais malignas, neoplasias epiteliais malignas tipos especiais, neoplasias mesenquimais malignas (sarcomas), carcinossarcoma, neoplasias benignas, hiperplasia/displasia, neoplasias de mamilo e hiperplasia/displasia de mamilo (GOLDSCHMIDT et al., 2011).

Entretanto as classificações disponíveis na literatura falham em identificar diferenças biológicas entre os subtipos histológicos, particularmente em pacientes com o mesmo tipo e grau neoplásico e mesma estrutura anatômica e morfológica (VARALLO et al., 2019). Além disso, devido aos diversos sistemas de classificação, os oncologistas têm encontrado dificuldade em selecionar aquele que melhor define e caracteriza a doença, bem como, a sua progressão (ZUCCARI et al., 2008).

A significância prognóstica dos tipos histológicos tem sido descrita por muitos pesquisadores (CLEMENTE et al., 2010) e as entidades histológicas associadas a pior prognóstico são o carcinoma anaplásico, o carcinoma sólido e o carcinossarcoma (CASSALI et al., 2014). Assim, o diagnóstico preciso, bem como a diferenciação entre neoplasias benignas e malignas, é importante para o sucesso dos protocolos de tratamento (KUMAR et al., 2020)

Paralelamente ao exame histopatológico, a técnica de imuno-histoquímica tem se mostrado uma importante ferramenta para a identificação de novos marcadores prognósticos e preditivos (PEÑA et al., 2014). Esta técnica permite a visualização, por cor, da ligação entre um anticorpo específico e um antígeno presente nas células tumorais (RAMOS-VARA, 2005; PEÑA et al., 2014) e assim os fenótipos celulares podem ser usados como uma ferramenta que contribui para o entendimento da biologia tumoral (ZUCCARI et al., 2008), sendo uma ferramenta eficaz no cenário clínico (VARALLO et al., 2019).

Na medicina humana existe um painel de marcadores estabelecido para uso no prognóstico clínico de mulheres com câncer de mama (ZAHA et al., 2014; DAI et al., 2016). Este painel determina a classificação fenotípica e inclui os receptores de estrogênio (ER) e progesterona (PR) e receptor do fator de crescimento epidérmico humano 2 (HER-2) (ZUCCARI et al., 2008; FERREIRA et al., 2009). Entretanto na medicina veterinária, existem poucos estudos atualmente com foco nessa classificação fenotípica e os resultados publicados são conflitantes (VARALLO et al., 2019).

A presença de receptores hormonais no câncer de mama de cadelas infere no controle e na dependência dos hormônios ovarianos no desenvolvimento, manutenção e evolução da doença. O estrógeno e a progesterona promovem alterações fisiológicas nas glândulas mamárias durante o ciclo estral e seus receptores estão presentes em tecidos normais e neoplásicos (GAMA; ALVES; SCHMITT, 2008). A expressão do receptor de progesterona, mediada pelo receptor de estrogênio, é geralmente verificada juntamente com a expressão de RE, sendo que o padrão de expressão do RP está associado com tempo livre de doença e sobrevida global (BARDOU et al., 2003). Nas fêmeas caninas, observa-se que a presença de RE é significativamente mais elevada em tumores benignos e a expressão do RP tende a diminuir progressivamente a partir de lesões hiperplásicas/displásicas, tumores benignos e malignos, assim, a diminuição da expressão de RE e RP tem relação com um pior prognóstico (NARDI et al., 2016).

O proto-oncogene C-erbB-2 ou também chamado HER-2/neu capaz de estimular o crescimento e a diferenciação de células epidérmicas e mesodérmicas, o aumento da expressão do HER2 em tumores específicos (HER2+) está associado à maior taxa de crescimento e proliferação das células neoplásicas, bem como ao desenvolvimento de metástases à distância e, conseqüentemente, a menor sobrevida das pacientes (MÉNARD et al., 2003). O que se verifica é a permanência constante do sinal que estimula a mitose, mediado pelo recrutamento das vias PI3K/AKT e AMPK (APPERT-COLLIN, et al., 2015).

Apesar de incipiente, a Biologia Molecular tem ampliado o conhecimento das alterações genéticas do câncer na veterinária (KUSEWITT, 2013). Através do conhecimento genômico, é possível identificar várias alterações envolvidas na patogênese da doença (TIMMERMANS-SPRANG et al., 2017) e também no prognóstico, pois fornecem informações acerca da origem do processo, presença ou não de receptores hormonais, proteínas indutoras ou inibidoras de apoptose e de oncogenes (SPOERRI et al., 2015).

O prognóstico da doença é incerto, tanto nas mulheres quanto nas cadelas, devido a heterogeneidade tumoral, caracterizada pela coexistência de diferentes clones de células neoplásicas que podem assumir desenvolvimentos distintos (COLOMBO, 2021). Avanços recentes foram feitos nos últimos anos para melhorar a compreensão das alterações moleculares em tumores caninos (KIM et al., 2019, VARALLO et al., 2019; KIM et al., 2020; COLOMBO et al., 2021) como resultado, o

painel genético e as propriedades moleculares dessas neoplasias tornaram-se mais claras e esclarecedoras.

1.3 Via PI3K/AKT/mTOR no câncer

As células se intercomunicam em um processo chamado sinalização extracelular, elas produzem moléculas específicas que se ligam a receptores específicos de outras células e ativam as vias de sinalização intracelular (MIRICESCU et al., 2021).

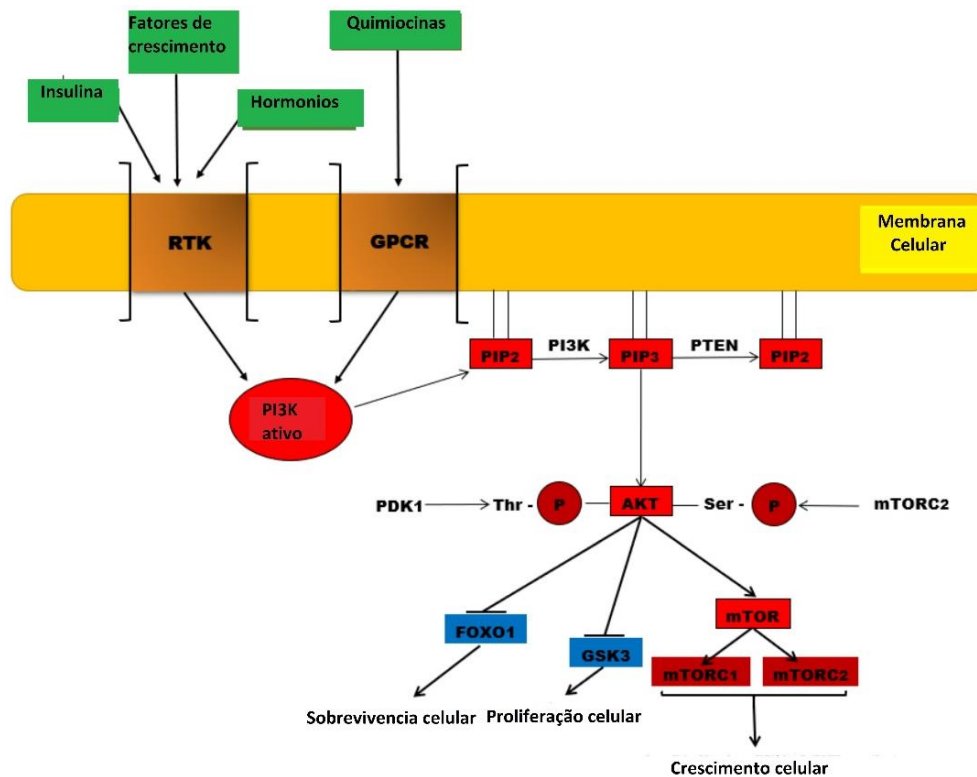
A fosfatidilinositol 3 quinase (PI3K) é uma enzima ligada à membrana plasmática ativada por receptores tirosina quinases (RTKs) e por receptores acoplados à proteína G (GPCRs), ambos pertencem a importantes famílias de receptores de superfície celular (LIM et al., 2005).

Os PI3Ks classe I são subdivididos em PI3Ks classe IA, PI3Ks classe IB e PI3Ks classe IC. Os IA-PI3Ks são heterodímeros, consistindo de uma unidade reguladora (p85 α , p85 β , p85 γ) que ativa a unidade catalítica (p110 α , p110 β , p110 δ , p110 γ), estão presentes em muitos tipos de tecidos e são ativados por receptores acoplados à proteína G (YU et al., 2015; YUDUSHKIN et al., 2019). Os PI3Ks IB são heterodímeros contendo uma subunidade reguladora (p101), que ativa a subunidade catalítica (p110 γ) (YU et al., 2015). A classe II PI3Ks tem três isoformas: PI3KC2 α e PI3KC2 β são expressos na maioria dos tecidos e órgãos, enquanto PI3KC2 γ é expresso apenas no fígado; eles regulam a dinâmica da membrana intracelular e o tráfego de membrana (BRACCINI et al., 2015). A classe III PI3Ks tem apenas um membro identificado: VPS34, que está ligado à regulação da fagocitose, pinocitose, classificação endossomal e autofagia (BACKER et al., 2016).

A sinalização PI3K/AKT, ilustrada na Figura 1, se inicia a partir da ativação das “receptor tyrosine kinases” (RTK), incluindo o Receptor do Fator de Crescimento Epidérmico (EGFR), o Receptor do Fator de Crescimento de Fibroblastos (FGFR) e o Receptor do Fator de Crescimento Semelhante à Insulina I (IGF-IR) (YANG et al., 2019). Os genes *PIK3R1* e *PIK3CA* codificam a subunidade reguladora p85 α , e a subunidade catalítica p110 α (THORPE et al., 2017), respectivamente. As RTKs ativadas recrutam os heterodímeros p85 α -p110 α para a membrana celular onde o fosfatidilinositol-4,5-bifosfato (PIP2) é catalisado por p110 α em trifosfato de fosfatidilinositol (PIP3) (SIEMPELKAMP et al., 2017). Em resposta ao recrutamento

de PIP3, a piruvato desidrogenase lipoamida quinase-1 (PDK1) ativa AKT e este, irá fosforilar outras substâncias como o complexo mTOR (YANG et al., 2019; MIRICESCU et al., 2021).

Figura 1 – Via de sinalização PI3K/AKT/mTOR. A PI3K é ativada pela ligação às RTKs e as “*G-protein-coupled-receptors*” (GPCR) de ligantes como insulina, fatores de crescimento e hormônios. Uma vez ativada, esta proteína quinase catalisará a fosforilação de PIP2 em PIP3. A AKT é recrutada para a membrana plasmática onde sofre dois processos de fosforilação, uma catalisada por PDK1 e a segunda catalisada por mTORC2. Uma vez ativada, a AKT irá fosforilar outras substâncias como o complexo mTOR, que será associado ao final com a síntese de proteínas e crescimento celular. Outros substratos fosforilados, como GSK-3 e FoxO1, serão inibidos, associados à proliferação e sobrevivência celular. PTEN é o principal regulador negativo desta via de sinalização envolvida na desfosforilação de PIP3.



Fonte: Modificado de MIRICESCU et al., 2021.

A via PI3K sofre muitas alterações no câncer, que são causadas por mutações ou ampliações de genes que codificam as subunidades catalíticas p110 α (PIK3CA) (YUDUSHKIN et al., 2019). Essas alterações genéticas podem causar modificações funcionais de proteínas que regulam as vias de sinalização intracelular, como a da PI3K/AKT/mTOR. Um exemplo importante é a via da fosfatidilinositol 3-quinase PI3K/AKT/mTOR, a qual desempenha papéis ativos em uma ampla gama de processos fisiológicos importantes, incluindo proliferação celular, sobrevivência, apoptose, motilidade, adesão, transformação e transporte de proteínas (KIM et al., 2019; KIM et al., 2021a).

Mutações no gene PIK3CA foram encontradas em diversas neoplasias na espécie humana, como glioblastoma, câncer gástrico, câncer de pulmão, câncer colorretal e câncer de mama (KIM et al., 2021a), sendo associadas com progressão e recidiva nessa classe tumoral (COLOMBO et al., 2021). Dessa forma, esse gene constitui um alvo molecular para novas intervenções terapêuticas (JURIC et al., 2019).

Com relação à espécie canina, diversas mutações somáticas já foram identificadas no gene PIK3CA, tais como c.1035T> A, c.1637A> C, c.1871C> A, c.3172A> T, c.3197C>T, c.3140A>G e a c.1035T>A, esta última, outra variante, menos comum, mas patogênica (LEE et al. 2019). Estudos recentes mostram que a mutação H1047R foi a mais encontrada no hemangiossarcoma canino (ALSAIHATI et al., 2020) e nos tumores mamários caninos (LEE et al., 2019; ALSAIHATI et al., 2020).

A mutação de ganho de função PIK3CA H1047R é conhecida por aumentar a atividade catalítica da enzima (KIM et al. 2021b), induzindo um ganho de função e rápida transformação e tumorigenicidade (MIRICESCU et al., 2021), promovendo a iniciação tumoral (ADAMS et al., 2011), desdiferenciação celular (KOREN et al. 2015), heterogeneidade tumoral e capacidade de invasão e migração em células de tumor mamário (DONG et al., 2017). Dessa forma, a superativação da via PI3K/AKT/mTOR pode interferir em várias vias de sinalização celular, e conseqüentemente, induzir a progressão do ciclo celular e proliferação celular e inibir a apoptose modulando a atividade dos membros da família "*B-cell lymphoma 2*" (Bcl-2) (BRUNELLE et al. 2009).

Da mesma forma que ocorre em mulheres, a identificação de mutações do gene PIK3CA em câncer de mama em cadelas pode resultar em novas abordagens terapêuticas, como, a utilização de inibidores de PI3K (MAVRATZAS et al., 2020). Na medicina veterinária existem poucos estudos explorando os padrões de expressão da via PI3K/AKT/mTOR em tumores mamários caninos (LEE et al. 2019; KIM et al. 2020; COLOMBO et al. 2021; KIM et al. 2021a;), bem como faltam pesquisas que analisem a correlação entre a mutação H1047R e a expressão de moléculas da via PI3K/Akt (KIM et al., 2021b), assim como se mostra relevante, a busca por métodos mais rápidos e precisos para identificar mutações em PIK3CA (WANG et al. 2021).

1.4A via PI3K/AKT/mTOR e a angiogênese

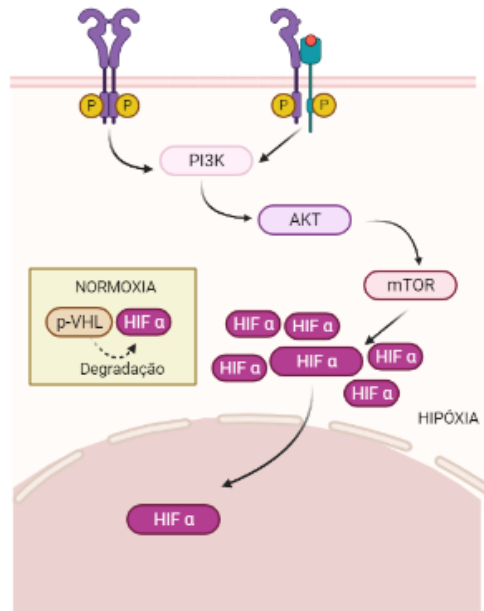
A angiogênese é o surgimento de novos ramos capilares da vascularização pré-existente e é indispensável para qualquer procedimento fisiológico e patológico, como desenvolvimento embrionário, regeneração tecidual, cicatrização de feridas, inflamação e até mesmo crescimento tumoral (DERYUGINA et al., 2015).

Comumente, as células endoteliais em circunstâncias fisiológicas normais são quiescentes; quando a demanda tecidual de nutrientes e oxigênio excede o habitual, como no caso de neoplasias, ocorre o surgimento de novos vasos procedentes de vasos sanguíneos preexistentes ou através da inserção de tecido intersticial no lúmen de vasos preexistentes (CARMELIET et al., 2000; WANG et al., 2019). Esse processo inclui a dissolução da membrana basal do vaso, migração e proliferação de células endoteliais, formação de um novo lúmen e ramos do vaso, maturação dos novos vasos pelo recrutamento de pericitos e a formação da membrana basal (JIANG et al., 2009).

A angiogênese tumoral pode ser desencadeada por sinais extracelulares, como fatores de crescimento, ou por alterações genéticas, como ativação de oncogenes, ou por mutações de genes supressores de tumor, como PTEN e p53 (JIANG et al., 2009).

A via PI3K/AKT/mTOR influencia o processo de angiogênese, contribuindo para formação de novos vasos sanguíneos como ilustrado na Figura 2; o Fator de crescimento endotelial VEGF e o Fator induzível por hipóxia (HIF-1) são os mediadores que transmitem sinais oncogênicos induzidos por PI3K/AKT para o crescimento tumoral e angiogênese (JIANG et al., 2009; XIE et al., 2019).

Figura 2 – PI3K na indução de angiogênese tumoral. PI3K é ativado por sinais extracelulares, como fatores de crescimento, ativação de tirosina quinases receptoras e oncogenes. PI3K induz a ativação de Akt, que aumenta a expressão do fator induzível por hipóxia-1 (HIF-1) através de mTOR. Fonte: elaborado pelo autor, 2022.



Fonte: Elaborado pelo autor

O HIF-1 é um fator de transcrição que regula a homeostase da concentração de oxigênio, composto por duas subunidades: HIF-1 α que pode ser induzido por hipóxia, fatores de crescimento e oncogenes e o HIF-1 β que é expresso constitutivamente em células humanas (SKINNER et al., 2004; WANG et al., 2019). Em condições normais de oxigênio, o HIF-1 α se liga à proteína Von Hippel-Lindau (p-VHL) e é degradado. Entretanto em condições de hipóxia, o HIF-1 α se acumula, e se transloca para o núcleo, regulando a transcrição de seus genes alvo, incluindo o VEGF, estimulando a formação de novos vasos sanguíneos e consequentemente a angiogênese tumoral (TANG et al. 2018). Estudos explorando a relação entre câncer de mama e HIF1 α em medicina veterinária são escassos, entretanto, alguns trabalhos relacionaram o aumento da expressão proteica de HIF1 α em tecido mamário de cadelas com prognóstico reservado (MADEJ et al., 2013; SHIN et al., 2015; MOTA et al., 2019; LI et al., 2021).

O gene supressor de tumor Von Hippel-Lindau (VHL) codifica uma proteína multifuncional, cujas mutações na linhagem germinativa predisõem os pacientes a vários tumores benignos e malignos altamente vascularizados estando, portanto, envolvido em diversas vias metabólicas como a angiogênese (GOSSAGE et al. 2015). Zhang et al. (2017) revelaram um atlas proteogenômico do câncer para alterações de

PI3K/AKT/mTOR que demonstra que as mutações de VHL estão associadas com sinalização AKT/mTOR altamente ativa, conferindo prognóstico mais reservado (ZHANG et al.; 2017). Portanto, a perda de VHL leva ao acúmulo de HIF α e consequentemente à ativação da cascata de angiogênese (ZHANG et al.; 2018). Sendo assim, a baixa expressão de VHL prejudica a degradação do HIF1 α , promovendo a ativação inadequada de genes alvo a jusante que normalmente seriam ativados apenas sob condições de hipóxia e, então, contribui diretamente para a tumorigênese, mais especificamente para a angiogênese. Dessa forma, quanto maior a expressão de VHL, melhor seria o prognóstico do paciente, uma vez que o VHL funciona como um supressor tumoral (LI et al. 2007; KNAUTH et al., 2006). Além do mecanismo da hipóxia, um estudo demonstrou que o “*Zinc Finger E-Box Binding Homeobox 1*” (ZEB1) promove a neoangiogênese do câncer de pulmão (CLARHAUT et al., 2009).

1.5A via PI3K/AKT/mTOR e metástase

Evidências têm mostrado que a transição epitelial-mesenquimal (EMT) está associada ao grau de malignidade em vários tipos de câncer, e acredita-se que a ativação da sinalização EMT contribua para invasão, metástase, recorrência e resistência terapêutica (ZHENG et al. 2015).

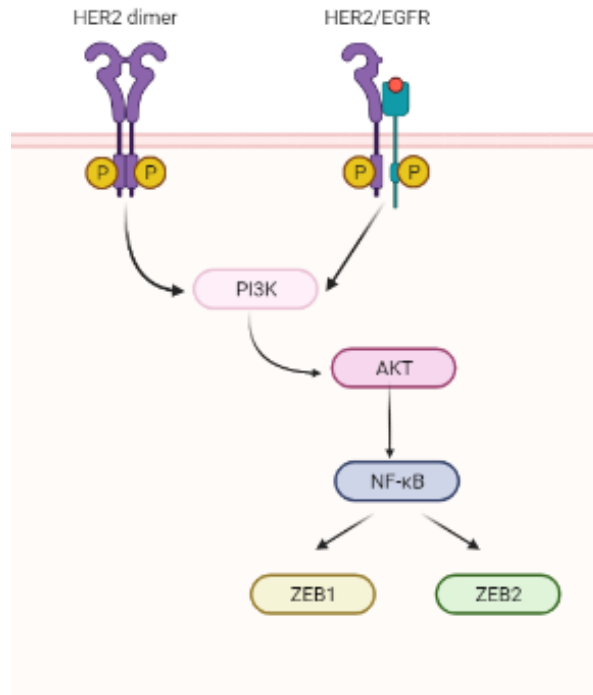
No processo de EMT, as células epiteliais são transformadas em um fenótipo mesenquimal invasivo, ocorrendo a perda da expressão da E-caderina (LARSEN et al. 2016); em contraste, as expressões de marcadores mesenquimais, incluindo vimentina (Vim) e N-caderina (N-cad), bem como fatores de transcrição relacionados a EMT, como Twist, a família Slug/Snail e ZEB1/ZEB2 (HE et al., 2020), são regulados positivamente, determinando um prognóstico clínico reservado na maioria das neoplasias (YUN et al., 2015; ZHANG et al., 2015).

A EMT é um processo reversível que é rigidamente regulado por vários fatores de transcrição como Twist, a família Slug/Snail e ZEB1/ZEB2 (ZHANG et al., 2019) sendo que a EMT controla a transcrição e tradução, mecanismos de estabilização de proteínas, *splicing* alternativo e expressão de RNAs não codificantes (SINGH et al., 2018). A família ZEB (ZEB1 e ZEB2) está envolvida na oncogênese por meio da via PI3K/AKT, e a ativação ou inibição dessa via promove ou inibe o processo de EMT (Figura 3) (WU et al., 2012).

O ZEB1 é um condutor essencial da progressão da ativação de EMT e tumorigênese para metástases avançadas, inibindo direta ou indiretamente os efeitos em alguns alvos cruciais a jusante, como a E-caderina (ZHANG et al., 2019). No processo de recolonização de células no câncer metastático, a via PI3K ativada de forma aberrante mostrou ter como alvo a ativação da transcrição de ZEB1 através da cascata de sinalização “*Glycogen synthase kinase-3*” (GSK3)/ β -catenina (WU et al., 2012); além dessa via, estudos mostram que mutação no gene “*Rat sarcoma virus*” (RAS) induz a transcrição de ZEB1 para inibir a expressão de PTEN (LIU et al., 2016). Outro estudo mostrou que a interleucina-17 (IL-17) estimula células de câncer e induz a expressão de ZEB1, que é mediada pela ativação da via de sinalização do “*nuclear factor kappa-light-chain-enhancer of activated B cells*” (NF- κ B), levando à perda da expressão de E-caderina e indução de EMT (Gu et al., 2015).

O gene, “*Zinc Finger E-Box Binding Homeobox 2*” (ZEB2), está à jusante da via PI3K/AKT/mTOR e também está envolvido no processo de EMT (WU et al., 2012). Estudos demonstram que a superexpressão de ZEB2 aumenta a progressão do tumor através da ativação das vias de sinalização do NF- κ B e PI3K/Akt em vários tipos de câncer humano, como o gástrico e mamário (HUANG et al., 2015; ZHANG et al., 2019b). Proteínas da família ZEB ligam-se à região promotora de genes relacionados à adesão celular, como a E-caderina, inibindo sua expressão transcricional (LARSEN et al., 2016; WU et al., 2019). Dessa forma, a alta expressão de ZEB1 e ZEB2 pode conferir um fenótipo pró-invasivo e um prognóstico reservado para a maioria das neoplasias (YUN et al., 2015; ZHANG et al., 2015).

Figura 3 – PI3K na indução de EMT através de ZEB1 e ZEB2. PI3K é ativado por sinais extracelulares e induz a ativação de Akt, que leva a ativação dos genes ZEB1 e ZEB2 através de mTOR, que promove o processo de transição epitélio mesenquimal. Fonte: elaborado pelo autor, 2022.



Fonte: Elaborado pelo autor

1.6A via PI3K/AKT/mTOR e apoptose

As caspases, são uma família de proteases específicas que atuam como mediadores da apoptose e esta família é dividida em duas classes: caspases iniciadoras, como a caspase-9, ativadas pelas vias de sinalização de apoptose, e caspases efetoras, como a caspase-3, que realizam apoptose (MAHESHWARI et al., 2011). A ativação da caspase-9 leva à liberação mitocondrial do citocromo c que por sua vez resulta na ativação da caspase-3 (LI et al., 2018).

A proteína caspase-3, codificada pelo gene CASP3, é localizada na membrana plasmática e citosol das células, e é um membro da via de sinalização das caspases e o mais importante executor da apoptose celular, sendo considerada a proteína mais prevalente nas células e responsável pela maioria dos efeitos apoptóticos, como a lise de proteínas importantes (LIN et al., 2016; LI et al., 2018)

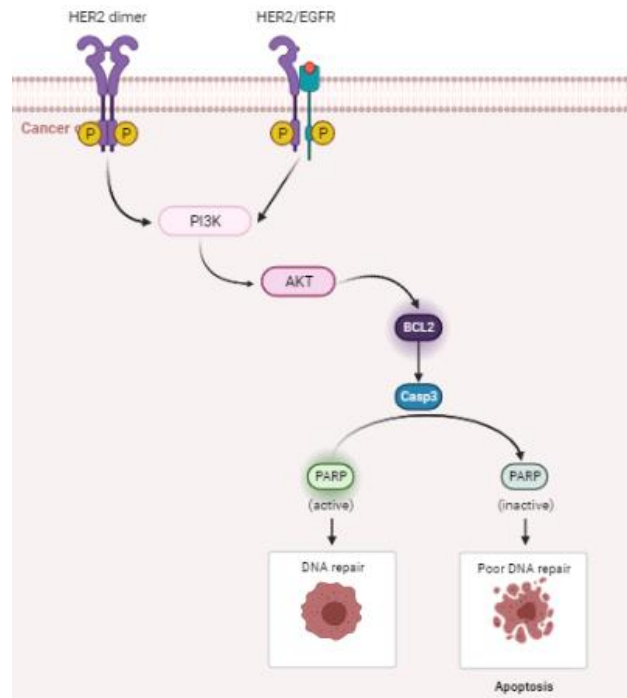
A via PTEN/PI3K/AKT constitui um importante eixo de regularização da sinalização da apoptose (LI et al., 2018; LI et al., 2019). Uma delas é pela ação da AKT que possui um potente sinal inibitório para a apoptose, tendo um papel importante na regulação da mesma (LI et al., 2018). A ativação de PI3K leva à fosforilação de AKT, que promove a sobrevivência celular (KANG et al., 2010).

A apoptose ainda pode ser inibida pela modulação da atividade dos membros da família Bcl-2 (CHANG et al., 2003). Para superar os sinais de estresse, as células neoplásicas frequentemente superexpressam as proteínas da família BCL-2, que são antiapoptóticas; conseqüentemente as caspases-3 conhecidas como "executoras" ou efetoras não realizam a clivagem proteolítica de substratos celulares específicos que resultam na morte celular (FERNALD et al., 2013).

Outro mecanismo que vem sendo estudado como alternativa para impedir a resistência das células tumorais aos quimioterápicos é o bloqueio da capacidade de reparação do DNA das células (PAPA et al., 2016). Proteínas específicas são ativadas em resposta a danos no DNA, dentre essas proteínas a poli (ADP-ribose) polimerase (PARP), a PARP1, que é a proteína mais abundante desta família, é considerada uma proteína nuclear que participa do mecanismo de reparo do DNA decorrente de alterações por quebra de fita simples do DNA (SSB). Esse mecanismo tem como finalidade manter a integridade genômica das células, reduzindo assim a sensibilidade das células tumorais aos quimioterápicos, garantindo-lhes resistência à terapia (BENAFIF and HALL, 2015; PAPA et al., 2016).

A ativação de AKT leva a fosforilação de uma proteína pró-apoptótica; que uma vez fosforilada, forma um complexo com a proteína citoplasmática adaptadora 14-3-3, desfazendo a heterodimerização com Bcl-xL e liberando-o para que exerça seu papel anti-apoptótico. Assim, ocorre a alteração do equilíbrio para a dominância de membros da família Bcl-2 anti-apoptóticos, estabilizando a integridade mitocondrial e promovendo a sobrevivência celular (Figura 4) (GALLYAS et al., 2020).

Figura 4 – PI3K e sua influência na via da apoptose celular. PI3K é ativado por sinais extracelulares e induz a ativação de Akt, que induz a proteína BCL-2 e consequentemente Caspase-3 e ativação de PARP1.



Fonte: Elaborado pelo autor

Na medicina humana o PARP1 vem sendo estudado para obtenção de novas opções de tratamento para mulheres que possuam mutações nos genes *Breast Cancer 1* e *2* (BRCA1 E BRCA2), através da aplicabilidade da teoria da letalidade sintética. Assim como PARP1, BRCA1/2 codificam proteínas envolvidas no reparo do DNA, com estes mutados e PARP1 inibido por meio de medicamentos, o reparo das fitas não pode ser realizado, o que acarreta na morte das células neoplásicas (KANIECKI et al., 2018).

Trabalhos correlacionando PARP1 com câncer na medicina veterinária são escassos. Donizy et al. (2020) observaram prognóstico reservado com o aumento de expressão de PARP1 em melanoma oral canino, com menor sobrevida dos animais. Thumser-Henner et al. (2020) relatam que até o momento não foram realizadas investigações aprofundadas sobre o uso de PARP1 em pacientes caninos com câncer de mama e mutação do gene BRCA2, exceto por Saba et al. (2016) que observaram que a utilização de iniparib, um inibidor de PARP1, em cães é tolerável, porém pouco efetivo.

1.7 O Antagonista PTEN

O gene "*phosphatase with tensin homology deleted in chromosome 10*" (PTEN) é uma fosfatase específica de *phosphatidylinositol-3, 4, 5-triphosphate* (PIP3) que desfosforila as moléculas de PIP3, resultando em moléculas de *phosphatidylinositol-4, 5-bisphosphate* (PIP2). Este não se liga a AKT, como consequência, AKT não pode ser ativado e a sinalização PI3K/AKT/mTOR é suprimida, atuando assim como supressor tumoral, inibindo a proliferação celular (NADERALI et al., 2018; LUONGO et al., 2019).

Devido seu papel na inibição da proliferação celular, o PTEN também atua na via do apoptose, na qual a diminuição da sua expressão causa superativação no processo anti-apoptótico (RESSEL et al., 2009; ASPRONI et al., 2021). Este sistema de sinalização contribui para o crescimento tumoral através da inibição da apoptose, neoangiogênese e aumento da capacidade metastática (SKINNER et al., 2004; JIANG et al., 2009; ASPRONI et al., 2021).

Em muitos tumores malignos, o gene PTEN sofre mutações, resultando em PTEN anormal, que não pode exercer seu efeito inibitório sobre a via PIP3/AKT/mTOR, ocorre então o aumento dos níveis plasmáticos de PIP3 e a atividade de Akt é continuamente estimulada (MIRICESCU et al., 2020).

A perda da função PTEN devido a mutações PTEN foi encontrada em 5-10% dos cânceres de mama (CARBOGNIN et al., 2019) e essa redução foi correlacionada com a graduação tumoral, status negativo do receptor de estrogênio e progesterona, metástase de linfonodo e prognóstico reservado (RESSEL et al., 2009).

As mutações PIK3CA foram associadas à positividade de ER e aumentaram com a idade, grau mais baixo e tamanho menor (ZARDAVAS et al., 2018). Na medicina veterinária, as alterações na expressão de PTEN foram investigadas em melanoma canino (KOENIG et al., 2002), hemangiossarcoma (DICKERSON et al., 2005), osteossarcoma (RUSSELL et al., 2018), câncer de próstata (CALDERÓN et al., 2016) e em tumores mamários caninos (BORGE et al., 2015) e carcinomas mamários felinos (MANISCALCO et al., 2012) confirmando o papel supressor entre diferentes espécies e neoplasias.

1.8 Justificativa

Devido a alta frequência de alterações da via PI3K/AKT/mTOR descritas no câncer de mama, o seu envolvimento em diversos processos celulares importantes, e

também pelo fato de existirem poucos estudos explorando os padrões de expressão da via PI3K/AKT/mTOR em tumores mamários caninos (LEE et al. 2019, KIM et al. 2021b; COLOMBO et al. 2021) torna-se de especial importância essa via, considerada um modelo importante para o estudo das proteínas expressas por genes que sejam estimulados por PIK3CA e PTEN, relacionados com metástase (ZEB1 e ZEB2), angiogênese (HIF-1 e VHL) e apoptose (CASPASE-3 e PARP1).

2 OBJETIVOS

2.1 Objetivo geral

Validar a variante c.3140A> G do gene PI3KCA e a expressão das proteínas alvo PI3K, HIF1 α , VHL, ZEB1, ZEB 2, Caspase 3, PARP1 e PTEN como marcadores prognósticos e preditivos que elegem as pacientes para a terapia de precisão.

2.2 Objetivos específicos

Avaliar a frequência da mutação PIK3CA c.3140A<G (H1047R) em determinada população canina por meio de RT-PCR e investigar a correlação entre a mutação, o prognóstico e as alterações a jusante da via, nas proteínas ZEB2, HIF1 α e PARP1, bem como as características histopatológicas.

Avaliar a expressão proteica e gênica de alvos moleculares à jusante da via PI3K/AKT/mTOR, bem como, investigar suas correlações com o prognóstico do câncer de mama canino.

3 MATERIAL E MÉTODOS

3.1 Estudo retrospectivo

3.1.1 Amostras

Foram utilizadas amostras de 58 cadelas adultas, de diferentes raças e idades, acometidas por neoplasia mamária, provenientes de um estudo prévio do nosso grupo de pesquisa. Estes fragmentos foram submetidos ao processamento histopatológico padrão e preservados em bloco de parafina. As cadelas foram acompanhadas durante 18 meses para a avaliação da ocorrência de recidivas e metástases.

O referido projeto foi aprovado pela Comissão de Ética em Experimentação Animal da Faculdade de Medicina de São José do Rio Preto (CEUA Licença 08/2019) da Faculdade de Medicina de São José do Rio Preto – SP (001-004391/2019) e realizado seguindo os padrões nacionais e internacionais de ética em experimentação animal.

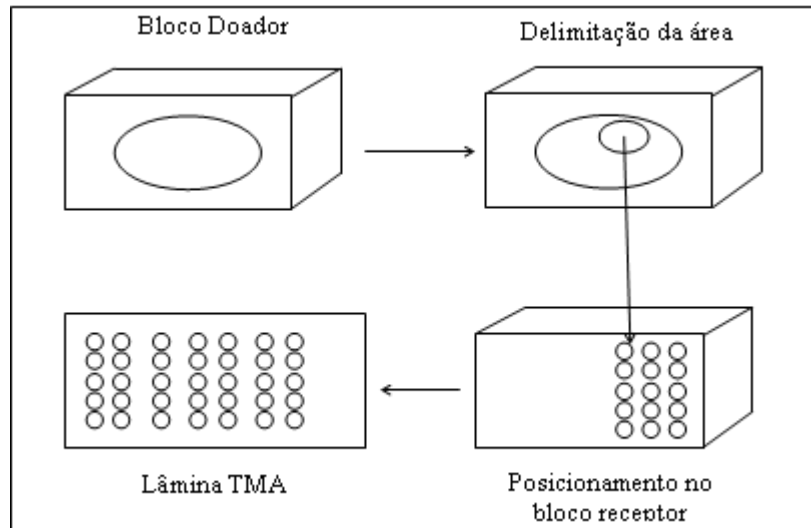
3.1.2 Análise da expressão das proteínas-alvo por imunohistoquímica

a) Tissue Microarray – TMA

A confecção do bloco de TMA parafinado foi realizada em projeto prévio (VARALLO, et al. 2018) e consistiu em uma coleta padronizada de amostras, todas processadas no mesmo laboratório pelo mesmo pessoal técnico, visando minimizar possíveis diferenças entre as amostras. Após processamento padrão, as lâminas histopatológicas, coradas em hematoxilina e eosina, foram analisadas por patologista veterinário (GMM) que delimitou uma região com maior representatividade e que apresentava os critérios de malignidade definidos no perfil da amostra. Nesses blocos doadores foram efetuados cortes com agulhas de 1,2 mm de diâmetro, nas áreas previamente marcadas, e os fragmentos cilíndricos foram depositados no bloco de parafina receptor (Figura 5).

Estes fragmentos cilíndricos são chamados na literatura de “*tissue core*” e neste trabalho vamos denominar *tissue core*, cada fragmento cilíndrico de cada amostra, portanto cada lâmina é constituída de amostras tumorais de diversas cadelas, identificadas em planilhas específicas.

Figura 5 – Ilustração da confecção da lâmina através do bloco de TMA. Esquema da técnica TMA. Após prévia revisão das lâminas originais e seleção das áreas de interesse para o estudo, faz-se a demarcação nos blocos de parafina arquivados (bloco doador). Em seguida, um cilindro de tecido é retirado do bloco doador com o auxílio de uma agulha acoplada a um equipamento de precisão. Após esse fragmento cilíndrico é introduzido em um novo bloco (bloco receptor). Os cilindros de várias amostras são sucessivamente adicionados no bloco receptor e a posição de cada caso é identificada em uma planilha.



Fonte: Adaptado de JAWAR, 2009.

a) Imuno-histoquímica

Os fragmentos de tecido das cadelas selecionadas foram submetidos a técnica de imuno-histoquímica para avaliação da expressão das proteínas-alvo. Iniciou-se com a desparafinização das lâminas em dois banhos de xilol por 5 minutos cada, seguido da reidratação em uma série de alcóois descendentes (dois absolutos, um 70 % e um 50 %) e banhos em água deionizada. Seguiu-se para a recuperação antigênica, em que as lâminas foram incubadas em tampão citrato (pH 6,0), pré aquecido (95 a 99 °C), por 30 minutos em panela a vapor. Logo após as lâminas permaneceram por 30 minutos em temperatura ambiente, para resfriarem e foram lavadas em solução tampão com fosfatos PBS (pH 7,0).

A partir deste passo foi utilizado o kit de revelação “REVEAL Polyvalent HRP-DAB Detection System”, da Spring Bioscience (Pleasanton, CA, USA) para todos os procedimentos, sendo realizados em câmara úmida. Primeiramente foi realizado o bloqueio da peroxidase endógena com a aplicação do “Hydrogen Peroxide Block” sobre os cortes e incubadas por 15 minutos, com posterior lavagem em tampão PBS por 5 minutos. Em seguida foi realizado o bloqueio de proteínas, com a incubação em “Protein Block” sobre os cortes por 10 minutos. Foi retirado o excesso desta solução

e, sem lavagem, aplicou-se o anticorpo primário (Tabela 2). Então as lâminas permaneceram incubadas por 18 horas a temperatura de 4 °C.

Tabela 2 – Lista de anticorpos, diluição e tecidos utilizados como controle positivo e negativo para as análises.

Anticorpo Primário	Diluição	Fabricado	Controle Positivo	Controle Negativo
Anti-PI3 Kinase Catalytic Subunit Alpha ab135958	1:50	Abcam Mouse monoclonal	Placenta	Fígado
Anti-HIF-1 α sc-53546	1:50	Santa Cruz Biot. Mouse monoclonal	Placenta	Fígado
Anti-VHL SAB4200285	1:300	Sigma-Aldrich Rabbit polyclonal	Kidney	Placenta
Anti-Zeb1 HPA027524	1:100	Sigma-Aldrich Rabbit polyclonal	Kidney	Placenta
Anti-Zeb2 SAB2108744	1:500	Sigma-Aldrich Rabbit polyclonal	Kidney	Placenta
Anti-Caspase 3 CP 229	1:50	BioCare Medical Rabbit polyclonal	Colon	Fígado
Anti-PARP SI258550	1:50	ThermoFischerMou se monoclonal	Baço	Fígado
Anti-PTEN SC-7974	1:100	Santa Cruz Biot. Mouse monoclonal	Placenta	Fígado

Após a incubação, os cortes foram lavados com o tampão de lavagem PBS. Para revelação, primeiramente os cortes foram incubados com o “Complement” (anticorpo secundário de coelho anti-rato) por 10 minutos e em seguida com o “HRP Conjugate” (anticorpo secundário de cabra anti-coelho conjugado ao HRP) por 15 minutos e então os cortes foram lavados com o tampão de lavagem PBS. Em seguida os cortes foram cobertos com diaminobenzidina (DAB) (20 μ l de “DAB Chromogen” diluído em 1 mL de “DAB Substrate”) por 3 minutos. Logo após a revelação, os cortes foram lavados com água deionizada, contracolorados com Hematoxilina de Harris por 40 segundos e novamente lavados em água deionizada. Por último, as lâminas passaram por uma série de concentrações de álcool e xilol e foram montadas com resina.

Todas as imunorreações foram acompanhadas de um controle positivo para o anticorpo testado e um controle negativo. Ao final do procedimento, a expressão proteica foi quantificada pela técnica de HistoScore.

HistoScore (H-Score) é uma medida para converter a imunohistoquímica clássica em valores quantitativos e é baseado na intensidade da coloração e nas porcentagens de células coradas e varia de 0 a 300, sendo dividido em quatro categorias de imunohistoquímica relatadas em células percentuais: células coradas negativas (0),

fracas (1+), moderadas (2+) e fortemente (3+). Em cada caso, um histoscore com uma faixa de potencial de 0-300 foi calculado da seguinte forma: $\text{HistoScore} = ((0 \times \% \text{ células não coradas}) + (1 \times \% \text{ células fracamente coradas}) + (2 \times \% \text{ células moderadamente coradas}) + (3 \times \% \text{ células fortemente coradas}))$ (JENSEN et al., 2017).

3.1.3 Forma de análise dos resultados

Para a realização da análise estatística procedeu-se ao uso do programa GraphPad Prism 8.0. Inicialmente, para avaliar o pressuposto de normalidade foi feito o teste de Shapiro-Wilk.

A curva ROC foi empregada de forma a decidir pelo *cut off* do índice de marcação da imuno-histoquímica que integra o melhor compromisso entre a sensibilidade e a especificidade, ou seja, quando estas estão o mais elevadas possível. Logo após, tendo este valor referido, foram determinadas as curvas de sobrevivência pelo método de Kaplan-Meier de modo a calcular as probabilidades cumulativas de sobrevivência entre os grupos de marcação positiva e negativa, seguindo-se o teste do logrank para comparação entre as curvas de sobrevivência. Um valor de $p < 0,05$ foi considerado estatisticamente significativo.

E por fim utilizou-se o teste de Correlação de Pearson (r) para avaliar as correlações entre a proteína PI3K e as demais proteínas realizadas e classificadas de acordo com os valores de Dancey e Reidy (2006).

3.2 Estudo Prospectivo (PCR)

3.2.1 Amostras

Foram selecionadas 24 pacientes caninas fêmeas, adultas, de diferentes raças, e idades, acometidas por neoplasia mamária, se tratando de casos primários e excluindo-se casos de recidiva, atendidas em clínicas veterinárias parceiras situadas na cidade de São José do Rio Preto e Catanduva, SP, (CEUA nº 001-004391/2019).

Esses fragmentos mamários foram enviados para o laboratório no qual o patologista definiu a região adequada para coleta da amostra. A técnica de escolha e acondicionamento seguiu um trabalho realizado por Raposo-Ferreira (2016), em que, da região selecionada, foram coletados fragmentos medindo aproximadamente $1 \times 1 \times 1 \text{ cm}^3$.

Para a coleta desses fragmentos, foram selecionadas regiões que não continham áreas de necrose perceptíveis pelo exame macroscópico e para seleção de amostras representativas para o exame histopatológico seguiu-se Araujo e Cassali (2017):

- Tumores menores que 3,0 cm: um fragmento;
- Tumores entre 3,0 e 5,0 cm: três fragmentos;
- Tumores maiores que 5,0 cm: cinco fragmentos.

Após selecionada a área, foi coletado o fragmento e dividido em dois, um para o exame histopatológico (acondicionado em solução de formalina a 10%) e outro para a análise molecular (armazenado em eppendorf estéril, previamente autoclavado, com a identificação do animal e localização na cadeia mamária, acondicionados em freezer -80 °C).

Esse procedimento, baseado no trabalho realizado por Weigelt et al. (2008), foi realizado para garantir, por meio da análise histopatológica prévia, que o tecido coletado para análise molecular possui tecido tumoral suficiente, assim como sua representatividade no diagnóstico tumoral.

Ainda, foram coletadas 20 amostras para constituir o grupo controle, obtidas de mamas de cadelas consideradas saudáveis ao exame clínico, tendo como critérios de exclusão, histórico prévio de neoplasia mamária, pseudociese ou qualquer outra lesão, como mamas de aspecto alterado no exame clínico (hiperemia, dor na palpação, secreção de material de qualquer natureza, presença de nodulações).

3.2.2 Análise Histopatológica

Para análise histopatológica, as amostras passaram por procedimento padrão até obtenção das lâminas coradas em HE e então classificadas segundo Goldschmidt et al. (2011) por médico veterinário patologista.

3.2.3 Extração de DNA dos fragmentos

A extração seguiu a técnica descrita por Sambrook & Russel (2001) com modificações e a extração foi realizada em duas etapas, primeiro extraindo RNA e depois o DNA.

As amostras foram maceradas com nitrogênio líquido e foi adicionado 1 mL de Trizol; em seguida adicionou-se 250 µL de clorofórmio e misturou-se por 1 minuto, incubando por 2 minutos a temperatura ambiente. Na sequência, os tubos foram centrifugados a 11.000 rpm por 15 minutos a 4 °C.

Após a centrifugação a amostra separou-se em três fases e para a extração do DNA, retirou-se o RNA sobrenadante e adicionou-se 300µL de etanol absoluto gelado. O material foi homogeneizado por inversão e incubado por 3 minutos a temperatura ambiente. Posteriormente, o material foi centrifugado a 11.000 rpm, a 4 °C por 10 minutos para a precipitação do DNA. O *pellet* de DNA formado após a centrifugação foi lavado duas vezes com solução de citrato de sódio 0,1M em etanol 10%. Para cada lavagem, o material foi deixado no agitador por 30 minutos a temperatura ambiente e depois centrifugado a 11.000 rpm, a 4 °C por 10 minutos. Depois da última lavagem, o pellet de DNA foi ressuscitado em 1,5 mL de etanol 75 % e mantido a temperatura ambiente por 15 minutos sob agitação periódica. Posteriormente, o material foi centrifugado a 11.000 rpm, a 4°C por 10 minutos. Em seguida, o sobrenadante foi descartado e o pellet foi seco a temperatura ambiente por 10 minutos. Após secagem do DNA, adicionou-se 50 µL de tampão TE e foi mantido em termomixer a 37 °C *overnight* para a completa diluição do DNA.

Após esse período foi realizada a leitura da concentração e do grau de pureza do DNA em espectrofotômetro. O restante do DNA foi armazenado a -20°C para posteriormente ser submetido às técnicas moleculares.

3.2.4 Escolha da sonda

Para a análise da mutação genética c.3140A>G foi verificada a homologia entre o gene PIK3CA humano e canino para verificar se havia equivalência entre as espécies e assim a sonda pudesse ser utilizada no presente trabalho.

A pesquisa foi realizada pelo site Ensembl e relacionou-se o segmento:

TTCATGAAACAAATGAATGATGCAC[**A/G**]TCATGGTGGCTGGACAACAAAAATG

Human CTGAGCAAGAGGCTTTGGAGTATTCATGAAACAAATGAATGATGCACATCATGGTGGCTGGACAACAAAAATG GATTGGATCTTCCACACAATTAACAGCATGCATTGAACTGAAAAG
Dog CTGARCAAGAGGCTTTGGAAATATTCATGAAACAAATGAATGATGCACATCATGGTGGCTGGACAACAAAAATG GATTGGATCTTCCACACCATTAAAGCAGCATGCTTTGAACTGAAATG

3.2.5 Análise de mutação PIK3CA H1047R por RT-PCR.

O DNA celular das linhagens MDA-MB-453 e T47D portando a mutação PIK3CA H1047R foram utilizadas como controle positivo para todas as análises de status de mutação PIK3CA H1047R, além da utilização do DNA de uma das pacientes positivas para a mutação encontradas em um estudo prévio de nosso grupo de pesquisa utilizando Sequenciamento de Próxima Geração (NGS) (COLOMBO et al., 2021).

A amplificação específica de alelo portando as mutações PIK3CA H1047R em amostras de DNA genômico canino foi realizada em um sistema de PCR em tempo real *Step One Plus (Applied Biosystems)*, como se segue. A PCR foi realizada com um PCR Premix Kit comercial (Maxime PCR PreMix Kit, iNtRON Biotechnology, Seul, Coréia). O conjunto de iniciadores (Forward: 50-CCCCAGAAGGCCTCTCTAAT-30; Reverso: 50-TGCAATCAGTCTTTGCCTGT-30) foi usado para detectar a mutação missense c.3140A> G (H1047R) na sequência de codificação de PIK3CA canino (referência NCBI: NC_006616.4). A condição de ciclagem de PCR foi a seguinte: desnaturação inicial a 95 °C por 10 min, 5 ciclos de 92 °C por 15 s, 58 °C por 1 min e por fim 40 ciclos de 92 °C por 15 s, 60 °C por 1 min.

A análise qualitativa de detecção de mutação foi realizada pelo programa *StepOne Software v2.3 (Applied Biosystems)*.

3.2.6 PCR quantitativo (qPCR)

O RNA total foi extraído dos fragmentos mamários pelo método TRIZOL (Invitrogen Life Technologies® - São Paulo, SP, Brasil), a concentração e a pureza do RNA total foram analisadas usando o espectrofotômetro NanoDrop 2000C (Thermo Fisher Scientific, Waltham, MA, EUA). O cDNA (fita simples - DNA complementar) foi sintetizado pelo Kit de cDNA de alta capacidade (Applied Biosystems®, Foster City, CA, EUA) em um volume total de 20 µL de misturas de reação na concentração final de 100 ng/µL. A expressão quantitativa foi realizada por PCR em tempo real em triplicata, utilizando o sistema *Step One Plus (Applied Biosystems®, Foster City, CA, EUA)*, e um controle negativo foi incluído em cada reação. As reações de PCR continham 100ng de cDNA, 10µL de TaqMan Universal Master Mix, 8µL de solução DEPC de água, 1µL de TaqMan Gene Expression para PIK3CA, ZEB2, HIF-1 e PARP-1 que foram submetidos ao seguinte esquema de amplificação: 50 °C por 2 min, 95 °C por 10 min, seguido por 40 ciclos de 95 °C por 15 segundos e 60 °C por 1 min.

As sequências utilizadas como controle endógeno foram previamente descritas por Moschetta et al. (2019). Para a análise da expressão gênica dos alvos, foram utilizados os ensaios TaqMan (Applied Biosystems®, Foster City, CA, EUA): PIK3CA(Cf02705766_m1), ZEB2 (Hs00207691_m1), HIF-1α (Cf02741632_m1) e PARP-1 (Cf02630973_m1).

A quantificação relativa (RQ) foi realizada pelo método $2^{-\Delta\Delta C_t}$ ⁹⁰. A expressão relativa das amostras foi analisada usando o grupo controle como calibrador (RQ=1). Os resultados foram expressos em mediana de unidade de expressão relativa.

3.2.7 Imuno-histoquímica

Os fragmentos de tecido das cadelas selecionadas com a mutação foram submetidos a técnica de imuno-histoquímica, já citada, para avaliação da expressão das proteínas-alvo PI3K, ZEB2, HIF-1 α e PARP1 (Tabela 1)

3.2.8 Análise dos resultados

Os resultados foram submetidos previamente à análise descritiva para determinação da normalidade. A comparação de dois parâmetros foi realizada pelo teste T não paramétrico. Valores de $p < 0,05$ foram considerados significantes e todas as análises serão realizadas com auxílio do software *Graphpad Prism*.

4 RESULTADOS

4.2 Artigo I - Validation of the c.3140A>G variant and its potential PIK3CA/AKT/mTOR target proteins in mammary neoplasms

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Abstract

Breast cancer is the most prevalent type of tumor in women, with a high rate of mortality and morbidity. Canine breast tumors are also very common, and dogs are considered models for the study of this neoplasm, as they have many similarities to humans. The PIK3CA/AKT/mTOR pathway is strongly unregulated in cancer, with emphasis on the c.3140A>G mutation of the PIK3CA gene, which is a non-silent mutation that alters target protein expression, making it more active. This study aimed to analyze the c.3140A>G variant of the PIK3CA gene in fragments of canine mammary tumors (CMT) and to correlate with the expression of PIK3CA, PARP-1, ZEB2 and HIF1 α proteins, which are stimulated by the pathway, as well as with the prognosis of the female dogs. DNA from 24 CMT fragments and 20 canine mammary normal fragments were extracted. The mutation was verified using TaqMan® Mutation Detection Assays. Immunohistochemistry was performed with the Reveal HRP Conjugate kit (Spring®) and its analysis, using the HistoScore method. Two patients (8.3%) showed the c.3140A>G mutation; one of them had pulmonary metastasis and died within 30 days of diagnosis, while the other patient remains in follow up after more than 180 days.

Immunohistochemistry data revealed that the expression of the target proteins was higher in the patient who died compared to the one who remains alive ($p < 0.05$). Thus, we can infer that, when the mutation c.3140A>G alters the expression of the PIK3CA protein as well as the expression of the PARP-1, ZEB2 and HIF1 α proteins, we can correlate with reduced overall survival.

Keywords: angiogenesis, apoptosis, H1047R, mammary cancer, metastasis.

INTRODUCTION

PIK3CA is the gene that encodes the p110 α catalytic subunit of class IA PI3K. Mutations or amplifications of this gene are frequently observed in solid tumors, especially breast cancer^{1,2}. Thus, this gene constitutes a molecular target for new therapeutic interventions¹.

Phosphatidylinositol 3-kinases (PI3Ks) are an important family of enzymes that participate in the regulation of a wide variety of important cellular processes, including cell proliferation, survival, apoptosis, motility, adhesion, morphology, transformation and protein transport^{3,4}.

The PIK3CA H1047R gain-of-function mutation is known to increase enzyme catalytic activity⁵, promoting tumor initiation⁶, cell dedifferentiation⁷, tumor heterogeneity⁸, in addition to invasiveness and migration capacity in breast tumor cells⁹. Thus, the overactivation of the PI3K/AKT/mTOR pathway can interfere with several cell signaling pathways, and consequently induce cell cycle progression and cell proliferation and inhibit apoptosis by modulating the activity of Bcl-2 family members¹⁰.

Regarding the canine species, several somatic mutations have already been identified in the PIK3CA gene, such as c.1035T>A, c.1637A>C, c.1871C>A, c.3172A>T, c.3197C>T, c. 3140A>G and c.1035T>A, another variant, less common but pathogenic¹¹.

In humans, mutations in the PIK3CA gene have been associated with progression and recurrence of breast cancer¹² and constitute a therapeutic target for precision therapy.

Just as it occurs in women, the identification of PIK3CA gene mutations in canine cancer in female dogs may result in new therapeutic approaches, such as the use of PI3K inhibitors¹³. Therefore, it is extremely important to search for faster and more accurate methods to identify mutations in PIK3CA¹⁴.

As mentioned before, the PI3K/AKT/mTOR cell signaling pathway involves several cellular processes. Thus, it is an important model for the study of expression of proteins expressed by genes that are stimulated by this cellular pathway, such as ZEB2, HIF1 α and PARP1. These genes participate in the processes of metastasis, angiogenesis and DNA repair, respectively.

The gene ZEB2 is downstream of the PI3K/AKT/mTOR pathway and is involved in the mesenchymal epithelial transition process (MET)¹⁵. Proteins from the ZEB family bind to the promoter region of genes related to cell adhesion, such as E-cadherin, inhibiting their transcriptional expression¹⁶. Thus, the high expression of ZEB2 can confer a pro-invasive phenotype and a reserved prognosis for most neoplasms^{17,18}.

Hypoxia-inducible factor (HIF-1) is a transcription factor that regulates the homeostasis of oxygen concentration, under normal oxygen conditions, HIF-1 α binds to the Von Hippel-Lindau protein (p-VHL) and is degraded. However, under hypoxic conditions, HIF-1 α accumulates, translocates to the nucleus, regulating the transcription of its target genes, including vascular endothelial growth factor (VEGF), stimulating the formation of new blood vessels and consequently tumor angiogenesis¹⁹. HIF-1 α is also regulated through the PI3K/AKT/mTOR pathway, which is activated by growth factors, including platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), epidermal growth factor (EGF), transforming growth factor (TGF), tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1B)²⁰.

The expression of HIF-1 α is increased when the PI3K/AKT/mTOR pathway is activated²⁰. Studies exploring the relationship between mammary cancer and HIF1 α in veterinary medicine are scarce, however, some studies have related the increased protein expression of HIF1 α in mammary tissue of female dogs with poor prognosis^{21,22,23,24}.

Specific proteins are activated in response to DNA damage. Among these proteins, poly (ADP-ribose) polymerase (PARP). PARP-1 is the most abundant protein in this family, being a nuclear protein that participates in the DNA repair mechanism resulting from alterations caused by DNA single-strand breakage (SSB). This mechanism aims to maintain the genomic integrity of cells, thus reducing the sensitivity of tumor cells to chemotherapy, ensuring resistance to therapy^{25,26}.

In this sense, the blocking of the DNA repair capacity of cells has been studied as an alternative to evade tumor cell resistance to chemotherapy²⁶. Studies correlating PARP-1 with cancer in veterinary medicine are scarce; however, Donizy et al. (2020)

observed a more reserved prognosis with increased PARP-1 expression in canine oral melanoma, with lower animal survival rates²⁷.

Considering the main regulatory functions of the PI3K/AKT/mTOR pathway, its common dysregulation in breast cancer and that there are few studies exploring the expression patterns of the PI3K/AKT/mTOR pathway in canine mammary tumors^{5,11,12,28} this work aimed to assess the frequency of the PIK3CA c.3140A<G (H1047R) mutation in a given canine population through RT-PCR and to investigate the correlation between the mutation, the prognosis and the alterations downstream of the pathway, in the ZEB2, HIF1 α and PARP1 proteins.

MATERIAL AND METHODS

Sample Collection and Histopathological Evaluation for RT-PCR

Twenty-four adult female canine patients of different breeds and ages, affected by primary breast cancer, and excluding cases of recurrence, were selected. As a control group, 20 samples were collected, obtained from mammary tissue of female dogs considered healthy on clinical examination. The breast fragments were obtained in partnership with veterinary clinics and a non-governmental organization – ONG. Exclusion criteria were: previous history of mammary cancer, pseudocystitis or any other lesion that demonstrates altered mammary on clinical examination. Histopathological evaluation, including histological grading and tumor subtypes, was performed based on the classification system of GOLDSCHMIDT et al. (2011)²⁹.

DNA extraction

The extraction followed the technique described by Sambrook & Russell (2001) with modifications, and the extraction was performed in two steps, first extracting RNA and then DNA³⁰.

After RNA extraction using the trizol technique, DNA extraction was performed with the two other reserved phases of RNA extraction, to which 300 μ L of cold absolute ethanol was added. The material was homogenized by inversion and incubated for 3 minutes at room temperature. Subsequently, the material was centrifuged at 13,000 rpm, at 4°C for 10 minutes for DNA precipitation. The DNA pellet formed after centrifugation was washed twice with 0.1M sodium citrate solution in 10% ethanol. For each wash, the material was left on the shaker for 30 minutes at room temperature and

then centrifuged at 13,000 rpm, at 4°C for 10 minutes. After the last wash, the DNA pellet was resuspended in 1.5 mL of 75% ethanol and kept at room temperature for 15 minutes with periodic shaking. Afterwards, the material was centrifuged at 13,000 rpm, at 4°C for 10 minutes. Then, the supernatant was discarded and the pellet was dried at room temperature for 10 minutes. After drying the DNA, 50µL of autoclaved ultrapure water was added and kept in a thermomixer at 37°C and 450 rpm, for 16 hours for complete DNA dilution. After this period, the reading of the concentration and degree of purity of the DNA was performed in a NanoDrop® (Thermo Fisher Scientific) device. The remainder of the DNA was stored at -20°C to be later submitted to molecular techniques.

Analysis of PIK3CA H1047R mutation by RT-PCR.

For the analysis of the c.3140A>G genetic mutation, the homology between the human and canine PIK3CA gene was verified for similarity between the species using Ensembl Genome Browser, being highly conserved. Cellular DNA from the MDA-MB-453 and T47D lineages carrying the PIK3CA H1047R mutation was used as a positive control for all PIK3CA H1047R mutation status analyses, in addition to using DNA from one of the mutation-positive patients found in a previous study by our research group using Next Generation Sequencing (NGS)¹².

Allele-specific amplification carrying the PIK3CA H1047R mutations in canine genomic DNA samples was performed on a Step One Plus real-time PCR system (Applied Biosystems) as follows. PCR was performed with a commercial PCR Premix Kit (Maxime PCR PreMix Kit, iNtRON Biotechnology, Seoul, Korea). The set of primers (Forward: 50-CCCCAGAAGGCCTCTCTAAT-30; Reverse: 50-TGCAATCAGTCTTTGCCTGT-30) was used to detect the missense mutation c.3140A>G (H1047R) in the canine PIK3CA coding sequence (NCBI reference: NC_006616.4). The PCR cycling condition was as follows: initial denaturation at 95°C for 10 min, 5 cycles of 92°C for 15 s, 58°C for 1 min and finally 40 cycles of 92°C for 15 s, 60°C for 1 min.

Qualitative mutation detection analysis was performed using StepOne Software v2.3 (Applied Biosystems).

Immunohistochemistry

Neoplastic tissue fragments from selected female dogs with the mutation and five normal tissues from female dogs were submitted to immunohistochemical technique to evaluate the expression of target proteins PI3K, ZEB2, HIF-1 α and PARP. The development kit “REVEAL Polyvalent HRP-DAB Detection System”, by Spring Bioscience (Pleasanton, CA, USA) was used for all procedures, being performed in a humid chamber. All immunoreactions were accompanied by a positive control for the antibody tested and a negative control (Supplementary Table 1).

At the end of the procedure, protein expression was quantified using the HistoScore (HS) technique. This is a measure to convert classical immunohistochemistry into quantitative values and is based on staining intensity and stained cell percentages, using ImageJ, and ranges from 0 to 300, being divided into four immunohistochemistry categories reported in percentage cells: negative stained cells (0), weak (1+), moderate (2+) and strongly (3+). In each case, a histoscore with a potential range of 0-300 was calculated as follows: $\text{HistoScore} = ((0 \times \% \text{ unstained cells}) + (1 \times \% \text{ weakly stained cells}) + (2 \times \% \text{ moderately stained cells}) + (3 \times \% \text{ strongly stained cells}))^{31}$. The HIF-1 α protein was also analyzed for the percentage of labeling.

Analysis of results

The results were previously submitted to descriptive analysis to determine normality. Comparison of two parameters was performed using the non-parametric t test between the neoplastic tissue and the mean values of the normal tissues. Values of $p < 0.05$ will be considered significant and all analyses will be performed using the *Graphpad Prism software*.

RESULTS

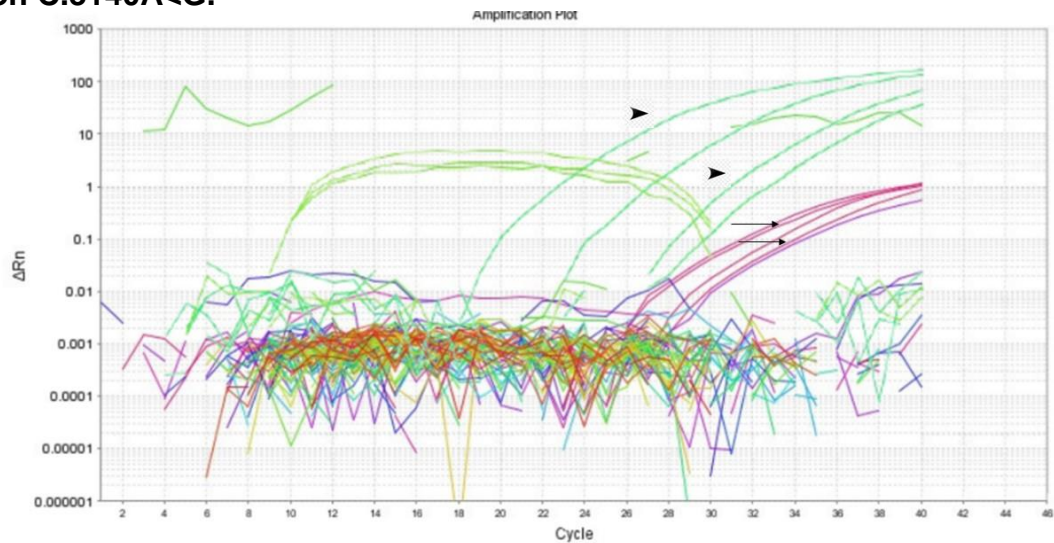
Analysis of PIK3CA H1047R mutation status in mammary cancer biopsy fragments.

Forty-four samples were analyzed; 20 from mammary tissue of female dogs without cancer, and 24 from neoplastic tissue. The age of the patients with mammary cancer ranged from 5 to 15 years, with a mean age of 10 years, with 7 spayed and 15 not spayed, most of them with no defined breed (12/24). As for the histopathological classification, there were: three complex grade I carcinomas, one lobular mammary

carcinoma, one mammary hemangiosarcoma, two grade I tubular carcinomas, three grade III tubular carcinomas, four grade II tubular carcinomas, two grade II complex carcinomas, one anaplastic carcinoma, one mammary osteosarcoma, one lobular mammary carcinoma, one undifferentiated carcinoma, one invasive lobular carcinoma, one benign mixed tumor, and two non-neoplastic lesions. Tumor classification, clinical features and histopathological results are available in Supplementary Table S2. The age of the control dogs ranged from 5 to 15 years, with a mean age of 10 years.

The analysis of the mutation of the PIK3CA gene, by real-time PCR, identified two samples (8.3%) (patients C18 and C24) with the H1047R mutation, while in the other samples the mutation was not observed (n=24). Samples were considered positive for the mutation when there was amplification following the positive control (Figure 1).

Figure 1. Amplification curve of mammary tumors in female dogs for point mutation C.3140A<G.



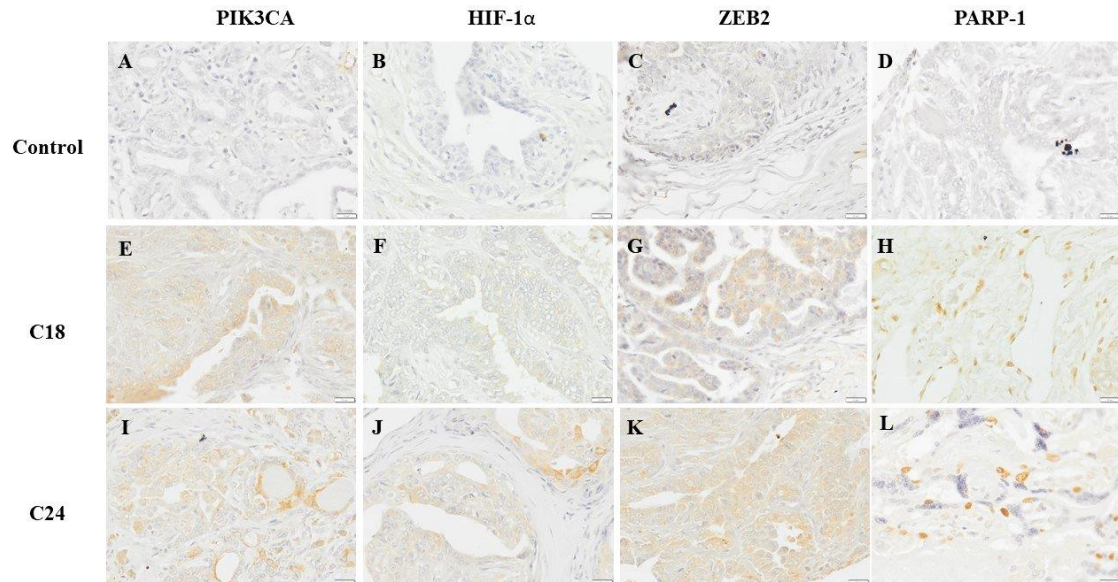
Subtitle: Sample amplification curve, showing the amplification of the positive controls (arrowhead) and samples C18 and C24 (arrows).

Immunohistochemistry

Two tumor fragments carrying the C.3140A>G mutation and a sample of normal mammary tissue from a female dog without a history of mammary alterations were analyzed. Immunohistochemistry was performed and immunostaining was considered positive when the cytoplasm of epithelial and stromal cells of tumor fragments were labeled for PIK3CA, ZEB2 and HIF-1 α and nuclear for PARP, with individual values for

proteins being C24 HS of 120, 174, 113 (<2%) and 118 and for the C18 HS of 102, 89, 90 (< 2%) and 93 respectively (Supplementary Table 3) as seen in Figure 2.

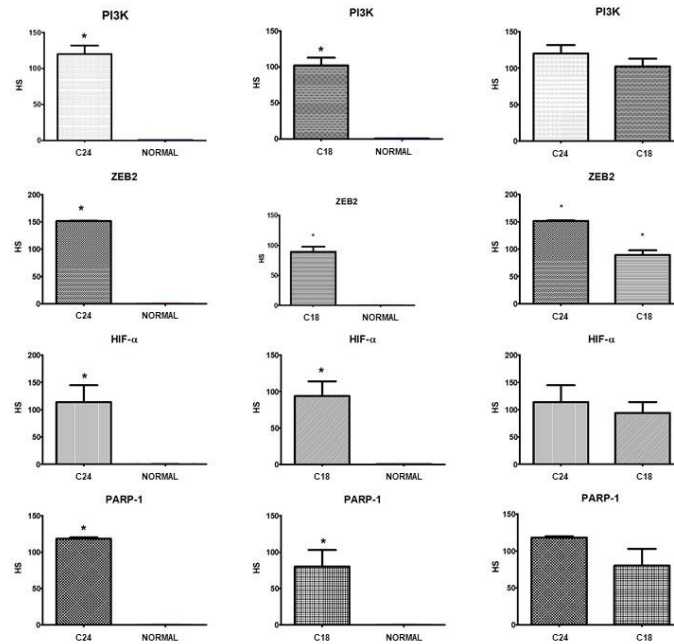
Figure 2. Photomicrographs of mammary tissue samples from C18 and C24 female dogs with carcinoma and control.



Subtitle: A/B/C/D) Negative expression of anti-PI3K p110 α antibodies; anti-HIF, anti-ZEB2 and anti-PARP in mammary tissue without neoplasia (Obj 20x). E/F/G) Cytoplasmic immunostaining of anti-PI3K p110 α antibodies; anti-HIF and anti-ZEB2 in mammary tumor of C18 (Obj 20x). H) Nuclear immunostaining of anti-PARP in mammary tumor of C18 (Obj 20x). I/J/K) Cytoplasmic immunostaining of anti-PI3K p110 α antibodies; anti-HIF and anti-ZEB2 in mammary tumor of C24 (Obj 20x). L) Nuclear immunostaining of anti-PARP in mammary tumor of C24 (Obj 20x).

Statistical analysis was performed with the results obtained from the C18 and C24 tumor samples, comparing with the results from the normal mammary sample. A statistically significant difference was observed for the correlations between C18 and normal and C24 and normal in relation to all evaluated proteins (PI3K, ZEB2, HIF and PARP), as well as for the correlation of C18 and C24 in relation to ZEB2 protein, as observed in Figure 3 ($p < 0.05$).

Figure 3. Statistical analysis of samples of mammary tumors in female dogs using the IHC technique.



Subtitle: Statistical analysis of tumor samples from mutated and dead female dogs (C24), mutated and alive patient (C18) and normal. There is a statistical difference between neoplastic and normal samples and also between C018 and C024 samples for ZEB2 protein ($p < 0.05$).

DISCUSSION

The PIK3CA/AKT/mTOR pathway is an important signaling pathway in humans and dogs. Saika et al. (2018) identified that among all somatic mutations found in human HER2-positive breast cancer, 24.2% were in PIK3CA, being significantly associated with ER-positive tumors while Levine et al. (2005) identified that 18% of the mutations found were in PIK3CA^{32,33}. In a study by Kim et al. (2020), the authors found that about 43.1% of canine mammary tumors had at least one non-silent mutation in genes of this pathway^{28,5}. Lee et al. (2019) also demonstrated that the PIK3CA gene was the most frequently mutated in canine mammary tumors, with the mutation verified in 29% of tumor samples¹¹. In both studies, the point mutation c.3140A<G was the mutation most frequently found in dogs.

In our study, we have found the mutation in 8,3% of canine mammary tumor samples; the difference with the data observed in the literature can be explained due to the method used. Garcia et al. (2018) observed that the PCR technique finds up to 5% of mutant alleles in a sample; however, the NGS technique may be more sensitive than that³⁴.

In the group of 24 patients with mammary tumors in our study, we verified two patients with the presence of the mutation, and in the histopathological analysis both had more aggressive carcinomas, unlike that observed in the study by Kim et al. (2021), who verified there was no association between the C.3140A>G mutation and the histological type⁵. Due to the small sample size of our work, further studies are needed to confirm an association between the expression of the studied proteins and the histological type of carcinoma.

The IHC results demonstrated that the presence of the mutation in PI3K was accompanied by overexpression of PI3K, ZEB2, HIF-1 α and PARP proteins in both patients. Furthermore, we observed that the patient with metastasis and death had higher histoscore values than the patient alive and without metastasis. This difference between the outcomes of female dogs can be explained by Kim et al. (2020), who observed that mutations in PIK3CA were frequently found in benign tumors of dogs, suggesting that the mutation is commonly acquired before malignant progression²⁸. Therefore, we observed that if there are changes in secondary pathway proteins, the prognosis is unfavorable. However, the limited number of patients evaluated does not allow defining a prognosis and should be evaluated in studies with a larger sample size.

Additionally, it has been proposed that there is a difference in the mechanisms between the pathway in women and in dogs. Kim (2021) observed that mutation in the PIK3CA gene in female dogs is associated with loss of PTEN and activation of AKT. These changes in women are related to increased risk of advanced breast cancer, resistance to hormonal treatment, increased risk of metastasis and poor prognosis⁵. However, this association was not observed in canine mammary tumors^{5,35,36,37}.

As for the IHC labeling for the PI3K protein, the classification of Aleskandarany et al. (2010), who evaluated the relationship of PI3K immunostaining and survival in women with breast cancer, was based on the HS, in which immunostainings above 101 were considered strongly positive³⁸. In our study, both canine patients had higher values. According to Aleskandarany et al. (2010), Garcia-Escudero et al. (2018) and Tapia et al. (2014) patients with PI3K-positive breast tumors, head and neck tumors and gastric tumors, respectively, had shorter survival and less disease-free time^{38,39,40}.

ZEB2 is associated with EMT and therefore is proposed to be involved in the progression of different types of tumors, such as breast cancer⁴¹. Other studies have also reported that co-expression of ZEB2 and other EMT-related protein markers is

associated with a poorer prognosis in human oral and head and neck squamous cell carcinoma^{41,42}. In our analyses we observed that patient C24, who had metastasis progression and death, had a high score (HS=174) while patient C18, who remains in follow-up after more than 180 days, had a low score (HS=89) ($p < 0.05$).

To define the ZEB2 protein cut off, we used data adapted from Qi et al. (2012), in which the cutoff point was $HS > 151$ ⁴³. In that study, the authors observed that ZEB2 was highly expressed in 65.6% of the glioma samples. A study by Zhu et al. (2018) also confirmed that ZEB2 protein expression was significantly increased in tumor tissue in human laryngeal squamous cell carcinomas, and was directly associated with the state of lymph node metastases and tumor cell differentiation, thus being considered a biomarker of EMT⁴⁴.

In a study carried out with five cell lines, two with epithelial morphology (E20 and E37), two with mesenchymal morphology (M5 and M25) and CF41, it was observed that ZEB2 is associated with greater aggressiveness in canine mammary carcinoma (CMC)⁴⁵. These results also support ZEB2 as a potential candidate for therapeutic targets for CMCs. Furthermore, they imply the possible use of dogs with spontaneous mammary tumors for the development of therapies based on the inhibition of EMT-related proteins⁴⁵.

Hypoxia is one of the most challenging conditions for cell survival and the strong expression of HIF-1 α may mean that neoplastic cells express HIF-1 α to survive a hypoxic environment²². For HIF1 α protein analysis, positive or negative labeling values were adapted from Kaya et al. (2012) and Peurala et al. (2012), where protein expression values $> 2\%$ were considered positive and $< 2\%$ as negative^{46,47}. In their analyses, both authors observed that a positive HIF1 α labeling was correlated with a lower overall survival in female patients, as also observed by Nalwoga et al. (2016)⁴⁸.

In a work carried out by SHIN et al. (2015) with mammary carcinomas in female dogs, it was observed that differences in HIF-1 α expression were significantly associated with the more aggressive histological type of CMTs ($P = 0.002$), a fact that can be compared with our patients, where both have histopathological types considered aggressive, such as undifferentiated carcinoma and mammary osteosarcoma. Madej et al. (2013) previously reported that tumor grade and HIF-1 α expression levels are significantly correlated with more aggressive mammary carcinomas^{21,22}.

Studies correlating PARP-1 and the PIK3CA pathway in canine breast cancer are relatively scarce in the literature, so we used reference values from studies in women. In the canine patients in our study, we observed low PARP-1 labeling when compared to Green et al. (2015) who used as a cut off marking >200 as positive⁴⁸. In a study carried out on gastric cancer, Liu et al. (2016) found that low and high expression levels of PARP-1 were defined as ≤ 175 and >175 , respectively, with high expression levels being associated with significantly shorter survival time⁵⁰.

Thus, the present study was able to identify the H1047R mutation of the PIK3CA gene in female dogs with mammary cancer. This mutation seems to increase the expression of PIK3CA protein and PIK3CA/AKT/mTOR pathway targets, ZEB2, HIF1 α and PARP1, as well as decrease overall survival time. There is an evident need for greater knowledge of changes related to the PIK3CA/AKT/mTOR pathway and its involvement in the molecular pathogenesis of canine breast tumors. The investigation of molecular alterations in this pathway can lead to new therapeutic targets in mammary cancer in female dogs, contributing to new treatment approaches.

References

1. Juric D, Janku F, Rodón J, Burris HA, Mayer IA, Schuler M, et al. Alpelisib Plus Fulvestrant in PIK3CA-Altered and PIK3CA-Wild-Type Estrogen Receptor–Positive Advanced Breast Cancer. *JAMA Oncology*. 2019;5(2):1-9
2. Pereira B, Chin S-F, Rueda OM, Vollan H-KM, Provenzano E, Bardwell HA, et al. The somatic mutation profiles of 2,433 breast cancers refine their genomic and transcriptomic landscapes. *Nature Communications*. 2016;7(1):1–16.
3. Cantley LC. The Phosphoinositide 3-Kinase Pathway. *Science*. 2002 May 31;296(5573):1655–7.
4. Bader AG, Kang S, Zhao L, Vogt PK. Oncogenic PI3K deregulates transcription and translation. *Nature Reviews Cancer*. 2005;5(12):921–9.
5. Kim S-H, Seung B-J, Cho S-H, Lim H-Y, Bae M-K, Sur J-H. Dysregulation of PI3K/Akt/PTEN Pathway in Canine Mammary Tumor. *Animals*. 2021;11(7):2079.
6. Adams JR, Xu K, Liu JC, Agamez NMR, Loch AJ, Wong RG, et al. Cooperation between *Pik3ca* and *p53* Mutations in Mouse Mammary Tumor Formation. *Cancer Research*. 2011;71(7):2706–17.
7. Koren S, Reavie L, Couto JP, De Silva D, Stadler MB, Roloff T, et al. PIK3CAH1047R induces multipotency and multi-lineage mammary tumours. *Nature*. 2015;525(7567):114–8.

8. Meyer DS, Koren S, Leroy C, Brinkhaus H, Müller U, Klebba I, et al. Expression of PIK3CA mutant E545K in the mammary gland induces heterogeneous tumors but is less potent than mutant H1047R. *Oncogenesis*. 2013;2(9):1–6.
9. Dong L, Meng F, Wu L, Mitchell AV, Block CJ, Zhang B, et al. Cooperative oncogenic effect and cell signaling crosstalk of co-occurring HER2 and mutant PIK3CA in mammary epithelial cells. *International Journal of Oncology*. 2017;51(4):1320–30.
10. Brunelle JK, Ryan J, Yecies D, Opferman JT, Letai A. MCL-1–dependent leukemia cells are more sensitive to chemotherapy than BCL-2–dependent counterparts. *Journal of Cell Biology*. 2009;187(3):429–42.
11. Lee K-H, Hwang H-J, Noh HJ, Shin T-J, Cho J-Y. Somatic Mutation of PIK3CA (H1047R) Is a Common Driver Mutation Hotspot in Canine Mammary Tumors as Well as Human Breast Cancers. *Cancers*. 2019;11(12):2006.
12. Colombo J, Moschetta-Pinheiro MG, Novais AA, Stoppe BR, Bonini ED, Gonçalves FM, et al. Liquid Biopsy as a Diagnostic and Prognostic Tool for Women and Female Dogs with Breast Cancer. *Cancers*. 2021;13(20):5233.
13. Mavratzas A, Marmé F. Alpelisib in the treatment of metastatic HR+ breast cancer with PIK3CA mutations. *Future Oncology*. 2020;17(1):13–36.
14. Wang G, Wu M, Durham AC, Mason NJ, Roth DB. Canine Oncopanel: A capture-based, NGS platform for evaluating the mutational landscape and detecting putative driver mutations in canine cancers. *Veterinary and Comparative Oncology*. 2021;
15. Wu K, Fan J, Zhang L, Ning Z, Zeng J, Zhou J, et al. PI3K/Akt to GSK3 β / β -catenin signaling cascade coordinates cell colonization for bladder cancer bone metastasis through regulating ZEB1 transcription. *Cellular Signalling*. 2012;24(12):2273–82.
16. Larsen JE, Nathan V, Osborne JK, Farrow RK, Deb D, Sullivan JP, et al. ZEB1 drives epithelial-to-mesenchymal transition in lung cancer. *The Journal of Clinical Investigation*. 2016;126(9):3219–35. Available from: <https://www.jci.org/articles/view/76725>
17. Yun E-J ., Baek ST, Xie D, Tseng S-F ., Dobin T, Hernandez E, et al. DAB2IP regulates cancer stem cell phenotypes through modulating stem cell factor receptor and ZEB1. *Oncogene*. 2015;34(21):2741–52.
18. Zhang P, Sun Y, Ma L. ZEB1: At the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance. *Cell Cycle*. 2015;14(4):481–7.
19. Tang Z, Xie H, Jiang S, Cao S, Pu Y, Zhou B, et al. Safflower yellow promotes angiogenesis through p-VHL/ HIF-1 α /VEGF signaling pathway in the process of osteogenic differentiation. *Biomedicine & Pharmacotherapy*. 2018;107:1736–43.
20. Xie Y, Shi X, Sheng K, Han G, Li W, Zhao Q, et al. PI3K/Akt signaling transduction pathway, erythropoiesis and glycolysis in hypoxia (Review). *Molecular Medicine Reports*. 2018;19(2):783–91.

21. Madej JA, Madej JP, Dziegiel P, Pula B, Nowak M. Expression of hypoxia-inducible factor-1 α and vascular density in mammary adenomas and adenocarcinomas in bitches. *Acta Veterinaria Scandinavica*. 2013;55(1).
22. Shin J-I ., Lim H-Y ., Kim H-W ., Seung B-J ., Sur J-H . Analysis of Hypoxia-Inducible Factor-1 α Expression Relative to Other Key Factors in Malignant Canine Mammary Tumours. *Journal of Comparative Pathology*. 2015;153(2):101–10.
23. Mota A de L, Jardim-Perassi BV, Castro TB de, Colombo J, Sonehara NM, Nishiyama VKG, et al. Melatonin modifies tumor hypoxia and metabolism by inhibiting HIF-1 α and energy metabolic pathway in the in vitro and in vivo models of breast cancer. *Melatonin-research.net*. 2019;2:83-98.
24. Li R, Wu H, Sun Y, Zhu J, Tang J, Kuang Y, et al. A Novel Canine Mammary Cancer Cell Line: Preliminary Identification and Utilization for Drug Screening Studies. *Frontiers in Veterinary Science*. 2021;8:1-12
25. Hall M, Benafif S. An update on PARP inhibitors for the treatment of cancer. *OncoTargets and Therapy*. 2015;8:519–28.
26. Papa A, Caruso D, Strudel M, Tomao S, Tomao F. Update on Poly-ADP-ribose polymerase inhibition for ovarian cancer treatment. *Journal of Translational Medicine*. 2016;14(1).
27. Donizy P, Wu C-L, Mull J, Fujimoto M, Chłópek A, Peng Y, et al. Up-Regulation of PARP1 Expression Significantly Correlated with Poor Survival in Mucosal Melanomas. *Cells*. 2020;9(5):1135.
28. Kim T-M, Yang IS, Seung B-J, Lee S, Kim D, Ha Y-J, et al. Cross-species oncogenic signatures of breast cancer in canine mammary tumors. *Nature Communications*. 2020;11(1):3616.
29. Goldschmidt M, Peña L, Rasotto R, Zappulli V. Classification and Grading of Canine Mammary Tumors. *Veterinary Pathology*. 2011;48(1):117–31.
30. Sambrook, J.; Russel, D.W. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press. 2001.
31. Jensen K, Krusenstjerna-Hafstrøm R, Lohse J, Petersen KH, Derand H. A novel quantitative immunohistochemistry method for precise protein measurements directly in formalin-fixed, paraffin-embedded specimens: analytical performance measuring HER2. *Modern Pathology*. 2016;30(2):180–93.
32. Saikia KK, Panigrahi MK, Mehta A, Kumar D. Clinico-pathological Features of PIK3CA Mutation in HER2-Positive Breast Cancer of Indian Population. *Indian Journal of Surgical Oncology*. 2018;9(3):381–6.

33. Levine DA, Bogomolny F, Yee CJ, Lash A, Barakat RR, Borgen PI, et al. Frequent Mutation of the PIK3CA Gene in Ovarian and Breast Cancers. *Clinical Cancer Research*. 2005;11(8):2875–8.
34. Alvarez-Garcia V, Bartos C, Keraite I, Trivedi U, Brennan PM, Kersaudy-Kerhoas M, et al. A simple and robust real-time qPCR method for the detection of PIK3CA mutations. *Scientific Reports*. 2018;8(1).
35. Perez-Tenorio G, Alkhori L, Olsson B, Waltersson MA, Nordenskjold B, Rutqvist LE, et al. PIK3CA Mutations and PTEN Loss Correlate with Similar Prognostic Factors and Are Not Mutually Exclusive in Breast Cancer. *Clinical Cancer Research*. 2007;13(12):3577–84.
36. Maruyama N, Miyoshi Y, Taguchi T, Tamaki Y, Monden M, Noguchi S. Clinicopathologic Analysis of Breast Cancers with PIK3CA Mutations in Japanese Women. *Clinical Cancer Research*. 2007;13(2):408–14.
37. Howlader, N et al. SEER cancer statistics review. National Cancer Institute. 2015, 2008, 1975–2012.
38. Aleskandarany MA, Rakha EA, Ahmed MAH, Powe DG, Paish EC, Macmillan RD, et al. PIK3CA expression in invasive breast cancer: a biomarker of poor prognosis. *Breast Cancer Research and Treatment*. 2009;122(1):45–53.
39. Tapia O, Riquelme I, Leal P, Sandoval A, Aedo S, Weber H, et al. The PI3K/AKT/mTOR pathway is activated in gastric cancer with potential prognostic and predictive significance. *Virchows Archiv*. 2014;465(1):25–33.
40. García-Escudero R, Segrelles C, Dueñas M, Pombo M, Ballestín C, Alonso-Riaño M, et al. Overexpression of PIK3CA in head and neck squamous cell carcinoma is associated with poor outcome and activation of the YAP pathway. *Oral Oncology*. 2018;79:55–63.
41. Chu P-Y, Hu F-W, Yu C-C, Tsai L-L, Yu C-H, Wu B-C, et al. Epithelial–mesenchymal transition transcription factor ZEB1/ZEB2 co-expression predicts poor prognosis and maintains tumor-initiating properties in head and neck cancer. *Oral Oncology*. 2013;49(1):34–41.
42. Kong YH, Syed Zanuuddin SN, Lau SH, Ramanathan A, Kallarakkal TG, Vincent-Chong VK, et al. Co-Expression of TWIST1 and ZEB2 in Oral Squamous Cell Carcinoma Is Associated with Poor Survival. Coleman WB, editor. *PLOS ONE*. 2015;10(7):1-18
43. Qi S, Song Y, Peng Y, Wang H, Long H, Yu X, et al. ZEB2 Mediates Multiple Pathways Regulating Cell Proliferation, Migration, Invasion, and Apoptosis in Glioma. Ahmad A, editor. *PLoS ONE*. 2012;7(6):e38842.
44. Zhu G, Song P, Zhou H, Shen X, Wang J, Ma X, et al. Role of epithelial–mesenchymal transition markers E-cadherin, N-cadherin, β -catenin and ZEB2 in laryngeal squamous cell carcinoma. *Oncology Letters*. 2018;15(3):3472–81.

45. Xavier PLP, Cordeiro YG, Rochetti AL, Sangalli JR, Zuccari DAPC, Silveira JC, et al. ZEB1 and ZEB2 transcription factors are potential therapeutic targets of canine mammary cancer cells. *Veterinary and Comparative Oncology*. 2018;16(4):596–605.
46. Kaya AO, Gunel N, Benekli M, Akyurek N, Buyukberber S, Tatli H, et al. Association of hypoxia inducible factor-1 alpha and carbonic anhydrase IX overexpression and survival in HER2/neu positive hormone-unresponsive breast cancer patients. *Journal of Clinical Oncology*. 2009;27(15):663-668.
47. Peurala E, Koivunen P, Bloigu R, Haapasaari K-M, Jukkola-Vuorinen A. Expressions of individual PHDs associate with good prognostic factors and increased proliferation in breast cancer patients. *Breast Cancer Research and Treatment*. 2011;133(1):179–88.
48. Nalwoga H, Ahmed L, Arnes JB, Wabinga H, Akslen LA. Strong Expression of Hypoxia-Inducible Factor-1 α (HIF-1 α) Is Associated with Axl Expression and Features of Aggressive Tumors in African Breast Cancer. Seagroves T, editor. *PLOS ONE*. 2016;11(1).
49. Green AR, Caracappa D, Benhasouna AA, Alshareeda A, Nolan CC, Macmillan RD, et al. Biological and clinical significance of PARP1 protein expression in breast cancer. *Breast Cancer Research and Treatment*. 2015;149(2):353–62.
50. Liu Y, Zhang Y, Zhao Y, Gao D, Xing J, Liu H. High PARP-1 expression is associated with tumor invasion and poor prognosis in gastric cancer. *Oncology Letters*. 2016;12(5):3825–35.

4.3 Material Suplementar Artigo 1

Supplementary Table S1. List of antibodies and tissues used as positive and negative controls.

PRIMARY ANTIBODY	DILUTION	PRODUCER	POSITIVE CONTROL*	NEGATIVE CONTROL*
Anti-PI3 Kinase Catalytic Subunit Alpha ab135958	1:50	Abcam Mouse monoclonal	Placenta	Liver (bile ducts)
Anti-HIF-1 α sc-53546	1:50	Santa Cruz Biot. Mouse monoclonal	Placenta	Liver
Anti-Zeb2 SAB2108744	1:500	Sigma-Aldrich Rabbit polyclonal	Kidney	Placenta
Anti-PARP SI258550	1:50	Thermo Fischer Mouse monoclonal	Spleen	Liver

Source: *The Human Protein Atlas, 2021.

Supplementary Table S2. Relationship between diagnoses and history of tumors in dogs.

ID	AGE	PSC	CASTRATED	AC	EVOLUTION	DIAGNOSIS
C01	-	-	-	-	-	Lobular Carcinoma Mammary
C02	10	No	Yes	No	> 6 months	Complex Carcinoma Grade I
C03	8	No	Yes	No	> 6 months	Mammary Hemangiosarcoma
C04	11	Yes	Yes	Yes	> 6 months	Complex Carcinoma Grade I
C05	9	No	No	Yes	> 6 months	Tubular Carcinoma Grade I
C06	-	No	No	No	1 month	Tubular Carcinoma Grade II
C07	7	No	No	No	< 6 months	Anaplastic Carcinoma
C08	13	No	No	No	< 6 months	Tubular Carcinoma Grade III
C09	-	No	No	Yes	> 6 months	Complex Carcinoma Grade II
C10	10	Yes	No	Yes	> 6 months	Tubular Carcinoma Grade II
C11	5	No	Yes	No	< 6 months	Tubular Carcinoma Grade III
C12	11	No	Yes	No	> 6 months	Tubular Carcinoma Grade II
C13	13	No	No	No	-	Complex Carcinoma Grade II
C14	13	No	No	No	-	Tubular Carcinoma Grade III
C15	7	No	No	Yes	> 6 months	Tubular Carcinoma Grade II
C16	12	No	No	No	> 6 months	Benign mixed tumor
C17	-	No	Yes	No	> 6 months	Complex Carcinoma Grade I
C18	6	-	-	-	-	Complex Carcinoma Grade II and mammary osteosarcoma

C19	13	No	No	No	> 6 months	Lobular mammary carcinoma in situ moderate grade
C20	-	No	No	No	> 6 months	Complex Carcinoma Grade I
C21	10	No	No	No	2 months	Tubular Carcinoma Grade II
C22	-	No	No	No	> 6 months	Invasive lobular carcinoma
C23	15	No	Yes	No	> 6 months	Ductal Mammary Carcinoma and Complex Carcinoma Grade II
C24	10	No	No	Yes	> 6 months	Undifferentiated Carcinoma

Legend: PSC: Pseudocyst. AC: Antiheat vaccine.

Supplementary Table S3. Relationship of Histscore (HS) results for antibodies used in immunohistochemistry.

ID	COMPLICATIONS	EVOLUTION	HS	
C18	No	Follow-up	PI3K	102
			ZEB2	89
			PARP-1	93
			HIF-1 α	90
C24	Metastasis	Death	PI3K	120
			ZEB2	174
			PARP-1	118
			HIF-1 α	113

4.4 Artigo II - Protein expression of PI3K/AKT/mTOR pathway targets validated by gene expression and its correlation with prognosis in canine mammary cancer

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Abstract

Breast cancer is the main type of neoplasia in female dogs, and is considered an adequate model for the biological and therapeutic study of cancer in women. The PIK3CA/AKT/mTOR pathway plays a central role in cellular homeostasis and is often dysregulated in cancer. The increased expression of PI3K protein in the literature is associated with a poor prognosis, and alterations in the PIK3CA gene can lead to changes in downstream pathways. Thus, the objective of this study was to validate the protein expression to confirm the gene expression of proteins belonging to the main pathway PI3K and PTEN, and their downstream pathways through ZEB1, ZEB2, HIF-1 α , VHL, CASPASE-3 and PARP1 relating to prognosis in canine mammary cancer. For protein studies, the samples came from 58 female dogs with mammary neoplasia, immunohistochemistry was performed and its analysis by the histoscore method. For the genetic evaluation, the samples came from 13 patients, the DNA was extracted and the analysis for quantitative expression. Through immunohistochemistry, PI3K positivity was significantly associated with affected regional lymph node, distant metastasis, patients with HER2+, Triple Negative and Luminal B phenotypes, and the lowest survival rates. Through gene expression, we observed higher gene expression of ZEB2 and PARP1 among patients who were alive and with death, which was not

true for the expressions of PIK3CA and HIF1 α . In conclusion, the data observed in this study are promising for the study of new molecular markers of prognosis suitable for the development of new therapies.

Key words: angiogenesis, apoptosis, H1047R, mammary cancer, metastasis.

INTRODUCTION

Breast cancer is the second leading cause of death for women worldwide [1]. Mammary cancer is also extremely common in companion animals such as dogs and cats [2]. There is great interest in mammary neoplastic changes in dogs due to the similarity of the behavior of the neoplasm in women, such as the high rate of recurrence, metastasis and mortality [3, 4].

In both species, the prognosis of the disease is reserved due to tumor heterogeneity, characterized by the coexistence of different clones of neoplastic cells [5]. Advances have been made in recent years to improve the understanding of molecular changes in canine tumors [5, 6, 7, 8]. As a result, the genetic panel and the molecular properties of these neoplasms have become clearer.

Genetic alterations can cause functional modifications of proteins that regulate intracellular signaling pathways, such as the PI3K/AKT/mTOR phosphatidylinositol 3-kinase pathway, which plays active roles in a wide range of important physiological processes. These include cell proliferation, survival, apoptosis, motility, adhesion, morphology, transformation and transport of proteins [6, 7].

Mutations in the PIK3CA gene have been found in several human cancers, such as glioblastoma, gastric cancer, lung cancer, colorectal cancer, and mammary cancer [7]. In veterinary medicine, mutations in this gene are often found; recent studies show that the H1047R mutation is the most frequently found in canine hemangiosarcoma [9] and canine mammary tumors [6, 7, 10].

The PTEN gene (phosphatase with tensin homology deleted in chromosome 10) is a tumor suppressor gene that functions as a negative regulator of cell proliferation [11, 12]. Alterations in this gene lead to failures in the signaling pathway and contribute to tumor growth through inhibition of apoptosis, neoangiogenesis and increased metastatic capacity [12, 13, 14]. In veterinary medicine, changes in PTEN expression have been investigated in canine melanoma [15], hemangiosarcoma [16], osteosarcoma [17], prostate cancer [18] and in canine mammary tumors [19] and feline

mammary carcinomas [20] confirming the suppressive role between different species and neoplasms.

To overcome the stress signals, neoplastic cells often overexpress the BCL-2 family proteins, which are anti-apoptotic. Consequently, the Caspases-3 known as "executors" do not carry out the proteolytic cleavage of specific cell substrates that result in cell death [21]. Other proteins researched in cancer are specific proteins activated in response to DNA damage. Among these proteins, poly (ADP-Ribose) polymerase (PARP), PARP1 is the most abundant protein of this family. In veterinary medicine, studies correlating PARP1 with cancer survival are scarce [22, 23, 24].

PI3K signaling also contributes to cell migration and migratory cell polarization in various cell types [25]. The "Zinc-finger factors" family (ZEB1 and ZEB2) is involved in oncogenesis through the PI3K/AKT pathway, in which its activation or inhibition promotes or suppresses the epithelial mesenchymal transition process (EMT) [26]. In the EMT process, they repress E-cadherin expression through direct binding in the promoter region, causing loss of E-cadherin expression and consequently promoting the development of metastatic properties, such as migration and invasion [27].

The PI3K/AKT/mTOR pathway also influences the angiogenesis process, contributing to the formation of new blood vessels. Hypoxia-inducible factor (HIF-1 α) is a transcription factor that regulates oxygen concentration homeostasis. HIF-1 α expression is increased when the PI3K/AKT/mTOR pathway is activated [28]. Under normal oxygen conditions, HIF-1 α binds to Von Hippel-Lindau (p-VHL) protein and is degraded, however, under hypoxic conditions, HIF-1 α accumulates and translocates to the nucleus, regulating the transcription of their target genes [29].

The loss of VHL leads to the accumulation of HIF-1 α and consequently the activation of the angiogenesis cascade [30]. Therefore, low VHL expression impairs the degradation of HIF-1 α , promoting inappropriate activation of target genes downstream [31]. Zhang et al. [30] revealed a cancer proteogenomic atlas for PI3K/AKT/mTOR alterations that demonstrates that VHL mutations are associated with highly active AKT/mTOR signaling, conferring a more guarded prognosis.

Therefore, due to the high frequency of alterations in the PI3K/AKT/mTOR pathway reported in mammary cancer, its involvement in several important cellular processes, and because there are few studies exploring the expression patterns of the PI3K/AKT/mTOR pathway in canine mammary tumors [5, 6, 7, 10], this pathway is considered an important model for the study of proteins expressed by genes that are

stimulated by PIK3CA and PTEN, related to metastasis (ZEB1 and ZEB2), angiogenesis (HIF-1 α and VHL) and apoptosis (CASPASE-3 and PARP1). Thus, this work aimed to evaluate the gene and protein expression of molecular targets downstream of the PI3K/AKT/mTOR pathway, as well as to investigate their correlations with the prognosis of canine mammary cancer.

MATERIAL AND METHODS

Retrospective study

Ethical considerations

This study was approved by the Animal Experimentation Ethics Committee (CEUA) (Protocols nº 3231/2012) of the Faculdade de Medicina de São José do Rio Preto – FAMERP.

Samples

Samples of 58 adult female canines of different breeds and ages, affected by mammary neoplasia, from a previous study by our research group were used [8]. These fragments were submitted to standard histopathological processing and preserved in a paraffin block. The female dogs were monitored for 18 months to assess the occurrence of recurrences and metastases. For the immunohistochemical analysis, the paraffin TMA block was used.

Immunohistochemistry

Tissue fragments from selected female dogs were submitted to immunohistochemistry technique to evaluate the expression of target proteins PI3K, PTEN, ZEB1, ZEB2, HIF-1 α , VHL, CASPASE-3 and PARP1. The “REVEAL Polyvalent HRP-DAB Detection System” development kit, from Spring Bioscience (Pleasanton, CA, USA) was used for all procedures, being performed in a humid chamber. All immunoreactions were accompanied by a positive control for the antibody tested and a negative control (Supplementary Table 1).

At the end of the procedure, protein expression was quantified by the Histoscore (HS) technique using the ImageJ program with the “Immunohistochemistry (IHC) Image Analysis” tool. HS is a measure to convert classical immunohistochemistry into quantitative values and is based on staining intensity and percentages of stained cells

and ranges from 0 to 300, being divided into four immunohistochemistry categories reported in percentage cells: stained negative cells (0), weak (1+), moderate (2+) and strong (3+). In each case, a histoscore with a potential range of 0-300 was calculated as follows: $\text{HistoScore} = ((0 \times \% \text{ unstained cells}) + (1 \times \% \text{ weakly stained cells}) + (2 \times \% \text{ moderately stained cells}) + (3 \times \% \text{ strongly stained cells}))$ [32].

Statistical analysis

To perform the statistical analysis, the GraphPad Prism 8.0 program was used. Initially, to assess the assumption of normality, the Shapiro-Wilk test was performed.

The ROC curve was used in order to decide on the cutoff of the immunohistochemical staining index that integrates the best compromise between sensitivity and specificity, that is, when they are as high as possible. Soon after having this value, the survival curves were determined by the Kaplan-Meier method in order to calculate the cumulative probabilities of survival between the positive and negative labeling groups, followed by the logrank test to compare the survival curves. A value of $p < 0.05$ was considered statistically significant.

Finally, the Pearson Correlation test (r) was used to evaluate the correlations between the PI3K protein and the other proteins performed and classified according to the values of Dancey and Reidy [33].

Prospective study

Ethical Considerations

This study was approved by the Animal Experimentation Ethics Committee (CEUA) (nº 001-004391/2019) of the Faculdade de Medicina de São José do Rio Preto – FAMERP.

Sample Collection and Histopathological Evaluation for RT-PCR

Thirteen adult female canine patients of different breeds and ages were selected, affected by primary mammary neoplasia and followed up for 365 days, patients with cases of recurrence, were excluded. As a control group, 5 samples were collected, obtained from the breasts of female dogs considered healthy on clinical examination. Exclusion criteria were: previous history of mammary cancer, pseudocyst or any other lesion that demonstrates breasts with an altered appearance on clinical examination. Histopathological evaluation, including

histological grading and tumor subtypes, was performed based on the classification system by Goldschmidt et al. [34] and can be observed in Supplementary Table 2.

Quantitative PCR (qPCR)

Total RNA was extracted from mammary fragments by the TRIZOL method (Invitrogen Life Technologies® - São Paulo, SP, Brazil), the concentration and purity of total RNA were analyzed using the NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA (single-stranded - complementary DNA) was synthesized by the High-Capacity cDNA Kit (Applied Biosystems®, Foster City, CA, USA) in a total volume of 20 µL of reaction mixtures at a final concentration of 100 ng/µL. Quantitative expression was performed by real-time PCR in triplicate using the Step One Plus system (Applied Biosystems®, Foster City, CA, USA), and a negative control was included in each reaction. PCR reactions contained 100ng of cDNA, 10µL of TaqMan Universal Master Mix, 8µL of DEPC water solution, 1µL of TaqMan Gene Expression for PIK3CA, ZEB2, HIF-1α and PARP1 which were subjected to the following amplification scheme: 50°C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 min.

The sequences used as endogenous controls have been previously described by Moschetta et al. (2019). For the analysis of the gene expression of the targets, the TaqMan assays (Applied Biosystems®, Foster City, CA, USA) were used: PIK3CA(Cf02705766_m1), ZEB2 (Hs00207691_m1), HIF-1α (Cf02741632_m1) and PARP1 (Cf02630973_m1).

Relative quantification (RQ) was performed using the $2^{-\Delta\Delta C_t}$ ⁹⁰ method. The relative expression of the samples was analyzed using the control group as a calibrator (RQ=1). Results were expressed as median relative expression unit.

RESULTS

Analysis of protein expression in mammary cancer samples in female dogs.

Clinicopathological characterization of the population

Fifty-eight samples of neoplastic mammary tissue from female dogs were analyzed and followed up for 540 days. At the end of this period, 44 patients remained in follow-up and 14 died, of which all had pulmonary and/or liver metastases and a mean survival of 274 days. These patients had been previously classified by

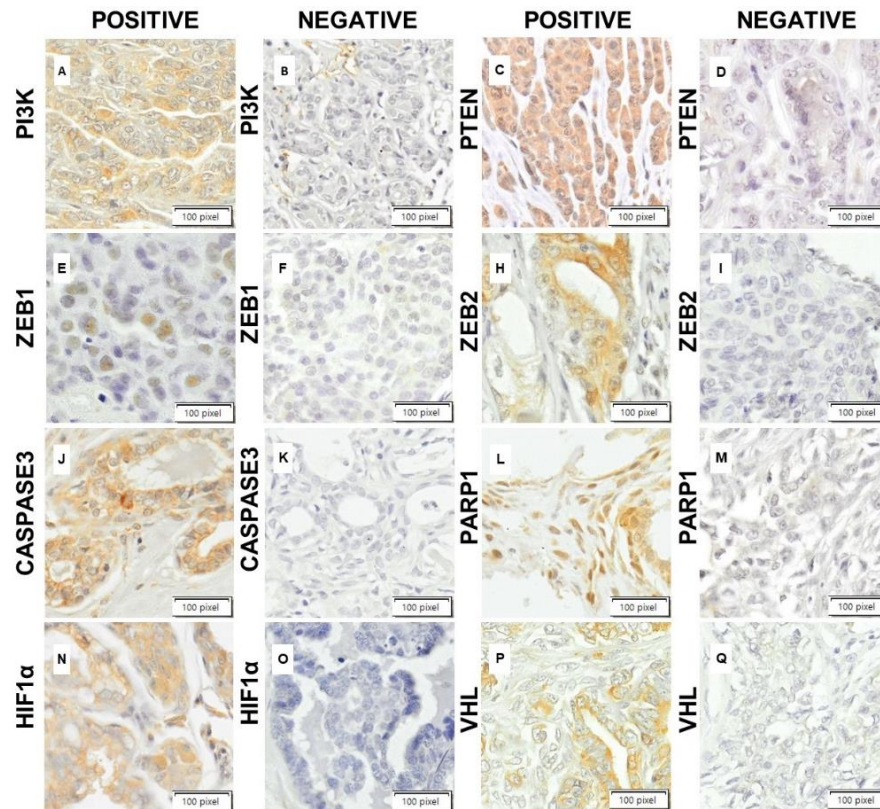
histopathological examination according to Goldschimit et al. [34], performed the TNM staging and analysis of the tumor phenotype shown in Supplementary Table 3, in addition to information on the presence of metastasis, death and survival in days.

In the histopathological analysis we observed: four carcinoma solid grade III, one carcinosarcoma, one mammary carcinoma in situ, one anaplastic mammary carcinoma, one tubular mammary carcinoma grade II, two micropapiliferous mammary carcinomas, one tubulopapiliferous mammary carcinoma grade I, four tubulopapiliferous mammary carcinomas grade II, five tubulopapiliferous mammary carcinomas grade III, eight complex mammary carcinomas grade I, thirteen complex mammary carcinomas grade II, nine complex mammary carcinomas grade III, one and two mammary carcinoma grade I, grade III respectively, without histopathological classification.

Immunohistochemistry (IHC) test analysis

Protein analyses were performed downstream of the PI3K pathway. IHC was performed on all 58 available tumor fragments, which revealed a median H-score for PI3K of 41, PTEN of 96, HIF-1 α of 25, VHL of 108, ZEB1 of 40, ZEB2 of 48, Caspase-3 of 92 and PARP1 of 40 (range 0 to 300). Fig 1 shows examples of staining results demonstrating positive and negative staining.

Fig 1. Photomicrographs of mammary tissue samples from patients.



Legend: A/C/H/J/N/P) Positive cytoplasmic immunostaining of anti-PI3K p110 α antibodies; anti-PTEN, anti-ZEB2, anti-Caspase3, anti-HIF-1 α and anti-VHL in mammary carcinomas (Bar 100). E/L) Nuclear immunostaining of anti-ZEB1 and anti-PARP1 in mammary carcinomas (Obj 10x). B/D/F/I/K/M/O/Q) Negative immunostaining of anti-PI3K p110 α antibodies; anti-PTEN, anti-ZEB1, anti-ZEB2, anti-Caspase3, anti-PARP1, anti-HIF-1 α and anti-VHL in mammary carcinomas (Bar 100).

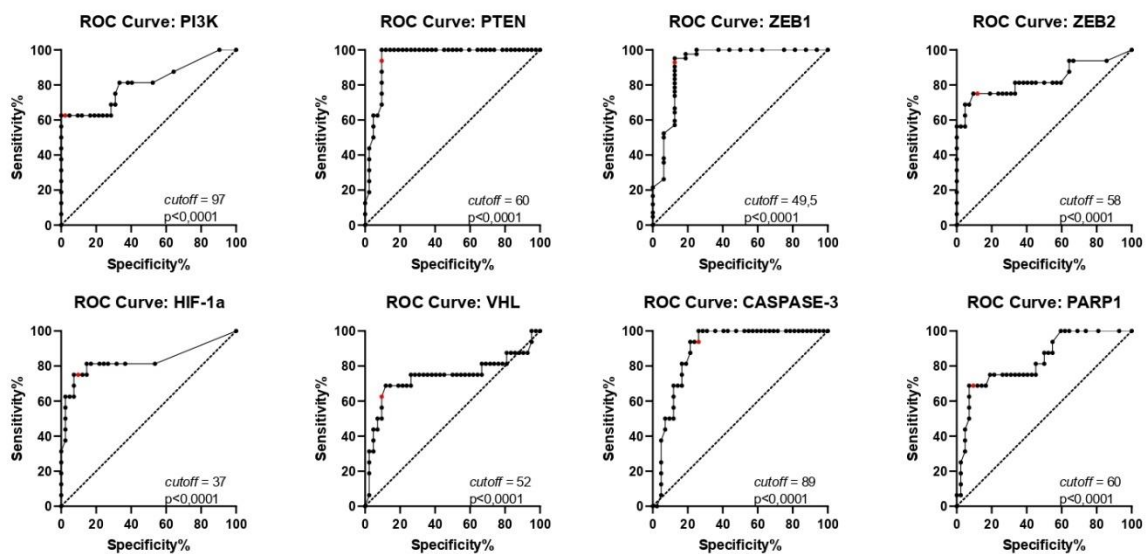
The cut-off points were determined from the Roc curve assays for each analyzed protein. Figure 2 shows the results; in all analyses $p < 0.05$. Among the mammary tumors, 17% ($n = 10$) were positive for PI3K. Among these, all patients progressed to the presence of metastasis and finally to death, in an average of 294 days. For its antagonist, PTEN, 43.5% ($n=20$) of the tumors studied were negative, with 85% ($n=17$) progressing to metastasis or recurrence and 70% ($n=14$) to death.

In the analysis of the HIF-1 α protein, 25.9% ($n=15$) of the tumors studied were positive, and only two patients (13%) remained alive and without metastasis. For VHL, 27.5% ($n=16$) were negative, of which only 18.7% ($n=3$) did not progress to metastasis and death. In the protein related to metastasis, for ZEB1, 29.3% ($n=17$) of the patients were positive. Of these, three remained alive and only one did not progress to metastasis or recurrence. For ZEB2, 27.5% ($n=16$) were positive. Of these, four remained alive without metastasis or recurrence. Regarding proteins related to the apoptosis pathway, Caspase-3 was 46.5% ($n=27$) positive, of which 51.8% ($n=14$)

evolved to death, and for PARP1, 24% (n=14) were positive; of these, only three patients did not die.

Regarding the analyzed proteins, the H-score and outcome values are recorded in Supplementary Table 3. In Supplementary Table 4, the best sensitivity and specificity values can be observed in relation to the cutoff value.

Fig 2. Receptor Operating Characteristic Curve (ROC) for assessing the discriminatory potential of proteins considering living and dying patients.



Legend: Labeling index ROC curves of proteins PI3K, PTEN, HIF-1 α , VHL, ZEB1, ZEB2, CASPASE3 and PARP1, for all $p < 0.0001$.

Association of PIK3CA with clinicopathological parameters.

Table 1 summarizes the associations between PI3K expression and clinicopathological variables. PI3K positivity was significantly associated with regional lymph node involvement and distant metastasis (Fisher test $p < 0.0001$). The positive expression of PI3K was not associated with tumor size and grade however it is important to emphasize that the tubulopapillary, complex, and solid tumor types were the most frequently observed in PI3K positive patients.

Table 1. Association between clinicopathological parameters of analyzed patients and positive or negative PI3K staining.

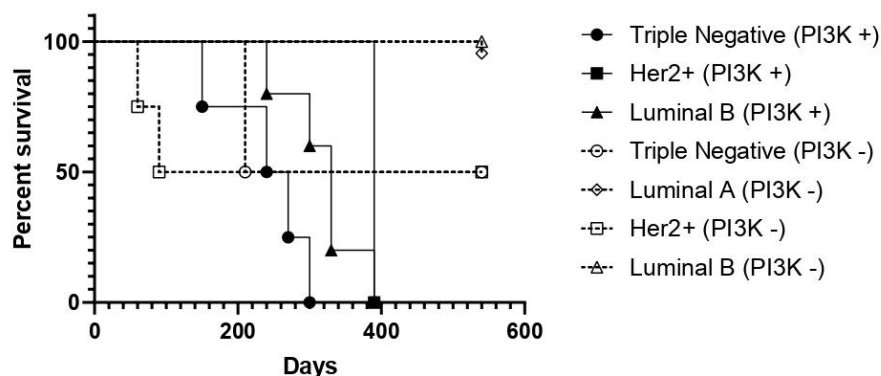
Parameters	Expression PI3K		Significance	
	Positive (%)	Negative (%)	χ^2	P value
Histopathological grade				
I e II	5 (8,6%)	29 (50,0%)		0,7261
III and specials	2 (8,6%)	19 (32,8%)		
Tumor Size				
T1	2 (3,5%)	23 (39,6%)		0,1626
T2 e T3	8 (13,8%)	25 (43,1%)		
Affected Regional Lymph Node				
Absent	2 (3,5%)	44 (77,2%)		0,0003
Present	6 (10,5%)	5 (8,7%)		
Distant metastasis				
Absent	0 (0%)	42 (72,4)		<0,0001
Present	10 (17,3%)	6 (10,3%)		

Association of PI3K in different immunohistochemically defined molecular subtypes of mammary cancer

The phenotypic characterization of these patients was previously performed by Varallo et al. [8]. In this study, it was observed that patients with HER2+, Triple Negative and Luminal B phenotypes associated with PI3K positivity resulted in a lower overall survival rate. In addition, all patients with Luminal A phenotypes were negative for PI3K, as seen in Figure 3.

Fig 3. Association between phenotype results and positive or negative staining of PI3K.

Survival proportions: Survival of Curva KM Fenótipos



Legend: Kaplan-Meier curve comparing the estimated survival, in days, and phenotypic profile of sick patients with negative and positive staining for PI3K ($p < 0.0001$).

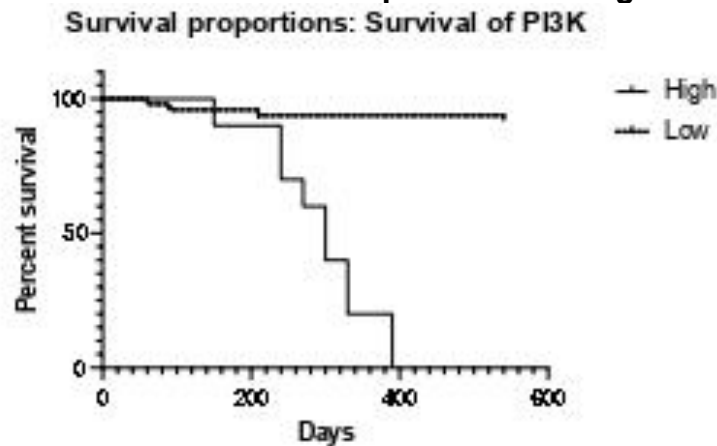
Association between PI3K and survival

The mean overall survival of patients was 475.9 days, with a minimum of 60 days and a maximum of 540 days (median 540 days and 95% CI). Univariate survival

analysis showed that patients with PI3K-positive mammary tumors had shorter survival [log rank (LR) = 50.26; $p < 0.0001$, Hazard ratio (HR) = 0.05314, 95% CI = 0.01043 to 0.2707; Figure 4].

Among the PI3K positive female dogs, all ten (10/10) died as a result of the disease. The negative patients were kept in follow-up, during which four female dogs (4/48) had metastasis or local recurrence without death, and three (3/48) died. The other patients (40/48) did not have complications associated with the disease in the 540 days of follow-up. The Supplementary Figure 1 shows the mean survival curve of patients for each protein studied.

Fig 4. Mean overall survival curve of PI3K positive and negative patients.



Legend: Kaplan-Meier curves comparing the estimated survival, in days, of sick patients with negative and positive labeling ($p < 0.0001$).

Association between PI3K and other proteins

Statistically applying Pearson's Correlation to evaluate the relationship of PI3K histoscore values with the histoscore values of the other proteins, we observed that PI3K has a moderate negative correlation with PTEN, a weak negative correlation with VHL and Caspase-3, and a moderate positive correlation with HIF-1 α , ZEB1, ZEB2 and PARP. The values of the correlation coefficient (r) and significance (p) can be observed in Supplementary Table 5.

Validation of canine mammary carcinoma markers by real-time RT-PCR.

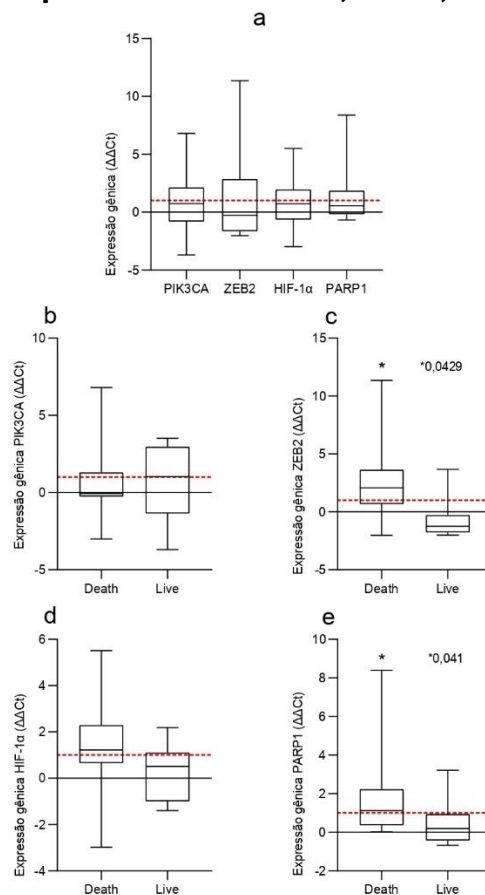
Gene expression of PIK3CA, ZEB2, HIF-1 α and PARP1

Real-time quantitative RT-PCR (qRT-PCR) was used to validate mammary tumor gene expression in female dogs by an independent method. Figure 4 illustrates

the expression levels of PIK3CA, ZEB2, HIF-1 α and PARP1 (Figure 5a). In this study, we did not observe overexpression of any of the genes when compared to the relative control (1.0; $p=0.6355$; $p=0.4973$; $p=0.5879$ and $p=0.5879$, respectively).

However, we observed higher gene expression of ZEB2 (Figure 5c) and PARP1 (Figure 5e) in patients who died (2.9 and 2.2) compared to those without metastasis (-0.5336 and 0.49; $p=0.0429$ and $p=0.041$ respectively). In addition, there was no significant difference between patients with mammary cancer who were alive or who died considering the expression of PIK3CA (0.63 and 0.78, respectively; $p=0.8873$ – Figure 5b) and HIF-1 α (0.31 and 1.3, respectively; $p=0.2075$ - Figure 5d).

Figure 5. Relative gene expression of PIK3CA, ZEB2, HIF-1 α and PARP1.



Legend: Relative gene expression of PIK3CA, ZEB2, HIF-1 α and PARP1 in patients with mammary carcinoma compared to the control group (a) and relative gene expression of these genes in patients with mammary carcinoma alive and dead (b, c, d and e).

DISCUSSION

The PI3K/AKT/mTOR signaling pathway participates in the regulation of processes such as growth, proliferation, survival, metastasis and chemotherapy resistance, thus participating in the promotion and progression of breast cancer [35].

In this study, genetic variants related to angiogenesis, survival and metastasis associated with the PI3K pathway were analyzed, aiming to clarify their association with gene and protein expression levels, tumor subtype, survival and metastasis in female dogs with mammary carcinoma.

In our study, we observed that all patients with increased protein expression of PI3K had a decrease in overall survival, progressing to metastasis and death. We also noted a positive correlation of high levels of PI3K, with lymphatic invasion and metastasis. However, we did not observe significant correlations between positivity of PI3K, tumor size and histopathological classification, results partially similar to those observed by [7], who did not observe correlation with affected lymph node.

The PI3K signal promotes the growth of estrogen receptor positive mammary cancer in an estrogen-independent manner [7]. In our analyses we observed a significant association between PI3K expression, not only between the Luminal molecular classes B and HER2+, but also for Triple Negative; results similar to those observed by Aleskandarany et al. [36] where HER2+, Luminal B and Triple Negative TN showed significantly increased proportions in cases of overexpression of PI3K.

In humans, Aleskandarany et al. [36], Garcia-Escudero et al. [37] and Tapia et al. [38], studying breast cancer, head and neck cancer, and gastric cancer respectively, observed that patients with PI3K-positive tumors had shorter survival and an association with shorter disease-free time. Thus, PI3K is an excellent candidate as a target for prognosis and therapy in canine mammary tumors. However, there are still few studies that correlate different clinicopathological parameters and their downstream pathways.

In the protein expression analysis of this study, all PI3K positive patients also had under-expressed PTEN. PTEN loss is reported in human and canine cancers, derived from epigenetic silencing, mutation and transcriptional repression [7, 39]. Studies carried out with feline mammary carcinoma observed that cats with loss of PTEN had a worse prognosis [11, 20]. Asproni et al. [12] observed that the expression of PTEN was inversely correlated with the malignancy of the neoplasm, being more expressed in adenomas than in canine carcinomas. Thus, the combination of PI3K and PTEN could be considered as a potential prognostic marker in mammary tumors.

In the literature, there is a relationship of overexpression of the PI3K/AKT/mTOR pathway with the angiogenesis-promoting pathway [28]. In this study, we observed a significant correlation between protein expression of PI3K and HIF-1 α , which

consequently causes a negative correlation of VHL. A significant correlation between PI3K and HIF-1 α was also observed by Sitaram et al. [40] in human kidney cancer. In the same work, they observed that the loss of PTEN also influences the loss of VHL. Studies exploring the relationship between mammary cancer and HIF1 α in veterinary medicine are scarce. However, some studies have related the increase in HIF-1 α protein expression in mammary tissue of female dogs with poor prognosis [41, 42, 43, 44].

In our study, we observed a significant positive correlation between PI3K and ZEB1 and 2, which corroborates studies found in the literature. In a study with metastatic bladder carcinoma, the authors observed that in the process of recolonization of neoplastic cells in bone tissue, the PI3K pathway was aberrantly activated, causing the activation of ZEB1 transcription [26]. In another study, also in humans, it was observed that the invasion of intrahepatic cholangiocarcinoma cells was mediated by the upregulation of ZEB1 through PI3K/AKT signaling [45].

With ZEB2, Wu et al. [46] observed that metastasis in lung cancer mediated by the downregulation of E-cadherin through the PI3K/AKT signaling pathway, decreased survival in these patients. In another study, they observed the existence of a PI3K/AKT-GSK-3 β -ZEB2 signaling pathway, which promotes IGF-I-induced EMT in gastric cancer cells, being considered an excellent clinical biomarker [47].

In this study, we observed a significant correlation between PI3K and the apoptosis-related proteins Caspase 3 and PARP1. The PI3K/AKT/mTOR lifetime pathway is widely studied in human works mainly in relation to therapy. An example is WANG et al. [48] who observed that the combination of PI3K and PARP1 inhibitory drugs resulted in reduced proliferation and significantly increased apoptotic cell death. In another study linking PARP1, Caspase 3 and PI3K with therapy and survival, the authors observed that the drug induced apoptosis in human osteosarcoma cancer cells via the PI3K/AKT pathway [49].

In the analyses of this work, there was a significant difference in the gene expression of ZEB2 and PARP1 between the groups of living and dead patients, confirming the data found in the retrospective study. The ZEB2 gene is a DNA-binding transcriptional regulator, which dimerizes with E-box in different promoters, such as E-cadherin, and negatively regulates this and other epithelial genes. Information available in the literature on the expression of ZEB2 in veterinary mammary cancer is scarce. However, Xavier et al. [50] observed a higher expression of ZEB2 in the canine

lineage CF41.Mg, the most malignant cell line in the experiment. These data corroborate our findings, in which patients with protein and gene overexpression of ZEB2 had a worse prognosis.

PARP is a nuclear protein responsible for the DNA repair process that can collaborate with tumor resistance [35]. The PARP gene and protein overexpression observed in this work are similar to those observed in the literature, where PARP is considered a marker for the repair process, being increased in cancer cells [51]. This result is promising, as this gene has been studied as a targeted therapy for mammary cancer in women and female dogs [52, 53].

Thus, according to our data, the ZEB2 and PARP1 genes may constitute new biomarkers of metastasis and survival in canine mammary cancer.

On the other hand, the gene expressions of PIK3CA and HIF-1 α did not reveal a significant difference between the groups of living and deceased patients, not confirming the alterations found in the retrospective study. HIF-1 α is a gene that controls angiogenesis and is constitutively expressed in human cells and, despite being scarce, there are studies that report increased expression in the mammary tissue of female dogs [44, 54] which was not observed in the gene expression of this study, even when comparing living and deceased patients.

PIK3CA, is considered a proto-oncogene related to several types of cancer, mainly in breast cancer [55], being actively studied in recent years in canine mammary cancer [5, 7, 10]. In this study, the data observed in gene expression were not similar to those observed in protein expression or to the data found in the literature. Thus, for the results of this study, PIK3CA gene expression does not define prognosis.

One of the reasons for the non-correlation of protein and genetic alterations in this work may be the fact that protein alterations do not always come from genetic alterations, because due to errors that accumulate over the various processes that occur from DNA to protein synthesis, gene expression may not interfere with protein synthesis [56]. In addition, our sample number for the analysis of gene expression was small, which may have compromised the results of this analysis. Thus, further studies with a larger sample size are interesting to clarify these results.

Therefore, by studying the protein expression of the patients, our results agree with the literature, in which alterations in the PIK3CA gene can lead to alterations in the downstream signaling pathways studied. However, for PIK3CA and HIF-1 α genes, this was not confirmed in the analysis of gene expression.

In conclusion, the data observed in this work are promising in the study of new molecular markers of prognosis suitable for the development of new therapies. Further research aimed at clarifying and validating the relationships between the PI3K/AKT/mTOR pathway and its downstream pathways are necessary to understand the progression of canine mammary carcinoma and confirm new molecular markers of suitable prognosis and for the development of new therapies.

References

1. Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, et al. Breast cancer. *Nature Reviews Disease Primers*. 2019 Sep 23;5(1). DOI: 10.1038/s41572-019-0111-2.
2. Nardi AB, Ferreira TMMR, Assunção KA (2016) Neoplasias Mamárias. In.: De Nardi AB, Daleck CR (Eds). *Oncologia de cães e gatos*. Rio de Janeiro: Roca, p. 726-756
3. Gelaleti GB, Borin TF, Maschio-Signorini LB, Moschetta MG, Hellmén E, Vilorio-Petit AM, et al. Melatonin and IL-25 modulate apoptosis and angiogenesis mediators in metastatic (CF-41) and non-metastatic (CMT-U229) canine mammary tumour cells. *Veterinary and Comparative Oncology*. 2017 Mar 20;15(4):1572–84. DOI: 10.1111/vco.12303.
4. Amirkhani Namagerdi A, d'Angelo D, Ciani F, Iannuzzi CA, Napolitano F, Avallone L, et al. Triple-Negative Breast Cancer Comparison with Canine Mammary Tumors From Light Microscopy to Molecular Pathology. *Frontiers in Oncology*. 2020 Nov 12; 10:563779. DOI: 10.3389/fonc.2020.563779.
5. Colombo J, Moschetta-Pinheiro MG, Novais AA, Stoppe BR, Bonini ED, Gonçalves FM, et al. Liquid Biopsy as a Diagnostic and Prognostic Tool for Women and Female Dogs with Breast Cancer. *Cancers*. 2021 Jan 1;13(20):5233. DOI: 10.3390/cancers13205233.
6. Kim K-K, Seung B-J, Kim D, Park H-M, Lee S, Song D-W, et al. Whole-exome and whole-transcriptome sequencing of canine mammary gland tumors. *Scientific Data*. 2019 Aug 14;6(1). DOI: 10.1038/s41597-019-0149-8.
7. Kim S-H, Seung B-J, Cho S-H, Lim H-Y, Bae M-K, Sur J-H. Dysregulation of PI3K/Akt/PTEN Pathway in Canine Mammary Tumor. *Animals*. 2021 Jul 1;11(7):2079. DOI: 10.3390/ani11072079.
8. Varallo G, Gelaleti G, Maschio-Signorini L, Moschetta M, Lopes J, De Nardi A, et al. Prognostic phenotypic classification for canine mammary tumors. *Oncology Letters*. 2019 Nov 5. DOI: 10.3892/ol.2019.11052.
9. Alsaihati BA, Ho K-L, Watson J, Feng Y, Wang T, Dobbin KK, et al. Canine tumor mutational burden is correlated with TP53 mutation across tumor types and breeds. *Nature Communications*. 2021 Aug 3;12(1):4670. DOI: 10.1038/s41467-021-24836-9.

10. Lee K-H, Hwang H-J, Noh HJ, Shin T-J, Cho J-Y. Somatic Mutation of PIK3CA (H1047R) Is a Common Driver Mutation Hotspot in Canine Mammary Tumors as Well as Human Breast Cancers. *Cancers*. 2019 Dec 12;11(12):2006. DOI: 10.3390/cancers11122006.
11. Ressel L, Millanta F, Caleri E, Innocenti VM, Poli A. Reduced PTEN Protein Expression and Its Prognostic Implications in Canine and Feline Mammary Tumors. *Veterinary Pathology*. 2009 May 9;46(5):860–8. DOI: 10.1354/vp.08-vp-0273-p-fl.
12. Asproni P, Millanta F, Ressel L, Podestà F, Parisi F, Vannozzi I, et al. An Immunohistochemical Study of the PTEN/AKT Pathway Involvement in Canine and Feline Mammary Tumors. *Animals*. 2021 Feb 1;11(2):365. DOI: 10.3390/ani11020365.
13. Jiang B, Liu L. Chapter 2 PI3K/PTEN Signaling in Angiogenesis and Tumorigenesis. Vol. 102, ScienceDirect. Academic Press; 2009. p. 19–65. DOI: 10.1016/S0065-230X(09)02002-8.
14. Skinner HD, Zheng JZ, Fang J, Agani F, Jiang B-H. Vascular Endothelial Growth Factor Transcriptional Activation Is Mediated by Hypoxia-inducible Factor 1 α , HDM2, and p70S6K1 in Response to Phosphatidylinositol 3-Kinase/AKT Signaling. *Journal of Biological Chemistry*. 2004 Oct;279(44):45643–51. DOI: 10.1074/jbc.m404097200.
15. Koenig A, Bianco SR, Fosmire S, Wojcieszyn J, Modiano JF. Expression and Significance of p53, Rb, p21/waf-1, p16/ink-4a, and PTEN Tumor Suppressors in Canine Melanoma. *Veterinary Pathology*. 2002 Jul;39(4):458–72. DOI: 10.1354/vp.39-4-458.
16. Dickerson EB, Thomas R, Fosmire SP, Lamerato-Kozicki AR, Bianco SR, Wojcieszyn JW, et al. Mutations of Phosphatase and Tensin Homolog Deleted from Chromosome 10 in Canine Hemangiosarcoma. *Veterinary Pathology*. 2005 Sep;42(5):618–32. DOI: 10.1354/vp.42-5-618.
17. Russell DS, Jaworski L, Kisseberth WC. Immunohistochemical detection of p53, PTEN, Rb, and p16 in canine osteosarcoma using tissue microarray. *Journal of Veterinary Diagnostic Investigation*. 2018 Apr 9;30(4):504–9. DOI: 10.1177/1040638718770239.
18. Rivera-Calderón LG, Fonseca-Alves CE, Kobayashi PE, Carvalho M, Drigo SA, de Oliveira Vasconcelos R, et al. Alterations in PTEN, MDM2, TP53 and AR protein and gene expression are associated with canine prostate carcinogenesis. *Research in Veterinary Science*. 2016 Jun 1; 106:56–61. DOI: 10.1016/j.rvsc.2016.03.008.
19. Borge KS, Nord S, Van Loo P, Lingjærde OC, Gunnes G, Alnæs GIG, et al. Canine Mammary Tumours Are Affected by Frequent Copy Number Aberrations, including Amplification of MYC and Loss of PTEN. Wade C, editor. *PLOS ONE*. 2015 May 8;10(5):e0126371. DOI: 10.1371/journal.pone.0126371.
20. Maniscalco L, Iussich S, Martín de las Mulas J, Millán Y, Biolatti B, Sasaki N, et al. Activation of AKT in feline mammary carcinoma: A new prognostic factor for feline mammary tumours. *The Veterinary Journal*. 2012 Jan;191(1):65–71. DOI:

10.1016/j.tvjl.2010.12.016.

21. Fernald K, Kurokawa M. Evading apoptosis in cancer. *Trends in Cell Biology*. 2013 Dec;23(12):620–33. DOI: 10.1016/j.tcb.2013.07.006.

22. Donizy P, Wu C-L, Mull J, Fujimoto M, Chłopik A, Peng Y, et al. Up-Regulation of PARP1 Expression Significantly Correlated with Poor Survival in Mucosal Melanomas. *Cells*. 2020 May 1;9(5):1135. DOI: 10.3390/cells9051135.

23. Thumser-Henner P, Nytko KJ, Rohrer Bley C. Mutations of BRCA2 in canine mammary tumors and their targeting potential in clinical therapy. *BMC Veterinary Research*. 2020 Jan 31;16(1). DOI: 10.1186/s12917-020-2247-4.

24. Saba C, Paoloni M, Mazcko C, Kisseberth W, Burton JH, Smith A, et al. A Comparative Oncology Study of Iniparib Defines Its Pharmacokinetic Profile and Biological Activity in a Naturally-Occurring Canine Cancer Model. Richards KL, editor. *PLOS ONE*. 2016 Feb 11;11(2):e0149194. DOI: 10.1371/journal.pone.0149194.

25. Cain RJ, Ridley AJ. Phosphoinositide 3-kinases in cell migration. *Biology of the Cell*. 2009 Jan;101(1):13–29. DOI: 10.1042/bc20080079.

26. Wu K, Fan J, Zhang L, Ning Z, Zeng J, Zhou J, et al. PI3K/Akt to GSK3 β / β -catenin signaling cascade coordinates cell colonization for bladder cancer bone metastasis through regulating ZEB1 transcription. *Cellular Signalling*. 2012 Dec 1;24(12):2273–82. DOI: 10.1016/j.cellsig.2012.08.004.

27. Larsen JE, Nathan V, Osborne JK, Farrow RK, Deb D, Sullivan JP, et al. ZEB1 drives epithelial-to-mesenchymal transition in lung cancer. *The Journal of Clinical Investigation*. 2016 Sep 1;126(9):3219–35. DOI: 10.1172/JCI76725.

28. Xie Y, Shi X, Sheng K, Han G, Li W, Zhao Q, et al. PI3K/Akt signaling transduction pathway, erythropoiesis and glycolysis in hypoxia (Review). *Molecular Medicine Reports*. 2018 Dec 3. DOI: 10.3892/mmr.2018.9713.

29. Tang Z, Xie H, Jiang S, Cao S, Pu Y, Zhou B, et al. Safflower yellow promotes angiogenesis through p-VHL/ HIF-1 α /VEGF signaling pathway in the process of osteogenic differentiation. *Biomedicine & Pharmacotherapy*. 2018 Nov 1;107:1736–43. DOI: 10.1016/j.biopha.2018.06.119.

30. Zhang P, Sun Y, Ma L. ZEB1: At the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance. *Cell Cycle*. 2015 Feb 16;14(4):481–7. DOI: 10.1080/15384101.2015.1006048.

31. Knauth K, Bex C, Jemth P, Buchberger A. Renal cell carcinoma risk in type 2 von Hippel–Lindau disease correlates with defects in pVHL stability and HIF-1 α interactions. *Oncogene*. 2005 Oct 31;25(3):370–7. DOI: 10.1038/sj.onc.1209062.

32. Jensen K, Krusenstjerna-Hafstrøm R, Lohse J, Petersen KH, Derand H. A novel quantitative immunohistochemistry method for precise protein measurements directly in formalin-fixed, paraffin-embedded specimens: analytical performance measuring

HER2. *Modern Pathology*. 2016 Oct 21;30(2):180–93. DOI: 10.1038/modpathol.2016.176.

33. Dancey CP, Reidy J. *Estatística sem matemática: para psicologia usando SPSS para Windows*. *Estatística sem matemática: para psicologia usando SPSS para Windows*. 2006;608–8.

34. Goldschmidt M, Peña L, Rasotto R, Zappulli V. Classification and Grading of Canine Mammary Tumors. *Veterinary Pathology*. 2011 Jan;48(1):117–31. DOI: 10.1177/0300985810393258.

35. Zhao Y, Cao J, Melamed A, Worley M, Gockley A, Jones D, et al. Losartan treatment enhances chemotherapy efficacy and reduces ascites in ovarian cancer models by normalizing the tumor stroma. *Proceedings of the National Academy of Sciences*. 2019 Feb 5;116(6):2210–9. DOI: 10.1073/pnas.1818357116.

36. Aleskandarany MA, Rakha EA, Ahmed MAH, Powe DG, Paish EC, Macmillan RD, et al. PIK3CA expression in invasive breast cancer: a biomarker of poor prognosis. *Breast Cancer Research and Treatment*. 2009 Aug 22;122(1):45–53. DOI: 10.1007/s10549-009-0508-9.

37. García-Escudero R, Segrelles C, Dueñas M, Pombo M, Ballestín C, Alonso-Riaño M, et al. Overexpression of PIK3CA in head and neck squamous cell carcinoma is associated with poor outcome and activation of the YAP pathway. *Oral Oncology*. 2018 Apr 1;79:55–63. DOI: 10.1016/j.oraloncology.2018.02.014.

38. Tapia O, Riquelme I, Leal P, Sandoval A, Aedo S, Weber H, et al. The PI3K/AKT/mTOR pathway is activated in gastric cancer with potential prognostic and predictive significance. *Virchows Archiv*. 2014 May 21;465(1):25–33. DOI: 10.1007/s00428-014-1588-4.

39. Milella M, Falcone I, Conciatori F, Cesta Incani U, Del Curatolo A, Inzerilli N, et al. PTEN: Multiple Functions in Human Malignant Tumors. *Frontiers in Oncology*. 2015 Feb 16;5. DOI: 10.3389/fonc.2015.00024.

40. Sitaram RT, Landström M, Roos G, Ljungberg B. Significance of PI3K signalling pathway in clear cell renal cell carcinoma in relation to VHL and HIF status. *Journal of Clinical Pathology*. 2020 May 28;74(4):216–22. DOI: 10.1136/jclinpath-2020-206693.

41. Madej JA, Madej JP, Dziegiel P, Pula B, Nowak M. Expression of hypoxia-inducible factor-1 α and vascular density in mammary adenomas and adenocarcinomas in bitches. *Acta Veterinaria Scandinavica*. 2013 Oct 24;55(1). DOI: 10.1186/1751-0147-55-73.

42. Li R, Wu H, Sun Y, Zhu J, Tang J, Kuang Y, et al. A Novel Canine Mammary Cancer Cell Line: Preliminary Identification and Utilization for Drug Screening Studies. *Frontiers in Veterinary Science*. 2021 May 27;8:665906. DOI: 10.3389/fvets.2021.665906.

43. Shin J-I., Lim H-Y., Kim H-W., Seung B-J., Sur J-H. Analysis of Hypoxia-Inducible Factor-1 α Expression Relative to Other Key Factors in Malignant Canine Mammary Tumours. *Journal of Comparative Pathology*. 2015;153(2):101–10. DOI: 10.1016/j.jcpa.2015.05.004.
44. Mota ADL, Jardim-Perassi BV, De Castro TB, Colombo J, Sonehara NM, Nishiyama VKG, et al. Melatonin modifies tumor hypoxia and metabolism by inhibiting HIF-1 α and energy metabolic pathway in the in vitro and in vivo models of breast cancer. *Melatonin Research*. 2019 Dec 15;2(4):83–98. DOI: 10.32794/mr11250042.
45. Liu L-Z, He Y-Z, Dong P-P, Ma L-J, Wang Z-C, Liu X-Y, et al. Protein tyrosine phosphatase PTP4A1 promotes proliferation and epithelial-mesenchymal transition in intrahepatic cholangiocarcinoma via the PI3K/AKT pathway. *Oncotarget*. 2016 Sep 19;7(46). DOI: 10.18632/oncotarget.12116.
46. Wu D, Zhang T, Liu Y, Deng S, Han R, Liu T, et al. The PAX6-ZEB2 axis promotes metastasis and cisplatin resistance in non-small cell lung cancer through PI3K/AKT signaling. *Cell Death & Disease*. 2019 Apr 25;10(5):1–15. DOI: 10.1038/s41419-019-1591-4.
47. Li H, Xu L, Zhao L, Ma Y, Zhu Z, Liu Y, et al. Insulin-like growth factor-I induces epithelial to mesenchymal transition via GSK-3 β and ZEB2 in the BGC-823 gastric cancer cell line. *Oncology Letters*. 2015 Jan 1;9(1):143–8. DOI: 10.3892/ol.2014.2687.
48. Wang D, Li C, Zhang Y, Wang M, Jiang N, Xiang L, et al. Combined inhibition of PI3K and PARP is effective in the treatment of ovarian cancer cells with wild-type PIK3CA genes. *Gynecologic Oncology*. 2016 Sep;142(3):548–56. DOI: 10.1016/j.ygyno.2016.07.092.
49. Chen Z-Z. Berberine Induced Apoptosis of Human Osteosarcoma Cells by Inhibiting Phosphoinositide 3 Kinase/Protein Kinase B (PI3K/Akt) Signal Pathway Activation. *Iranian Journal of Public Health*. 2016 May 1;45(5):578–85.
50. Xavier PLP, Cordeiro YG, Rochetti AL, Sangalli JR, Zuccari DAPC, Silveira JC, et al. ZEB1 and ZEB2 transcription factors are potential therapeutic targets of canine mammary cancer cells. *Veterinary and Comparative Oncology*. 2018 Jul 25;16(4):596–605. DOI: 10.1111/vco.12427.
51. Moschetta-Pinheiro MG, Colombo J, Godoy BLV de, Balan JF, Nascimento BC, Zuccari DAP de C. Modulation of Epithelial Mesenchymal Transition after AGTR-1 Gene Edition by Crispr/Cas9 and Losartan Treatment in Mammary Tumor Cell Line: A Comparative Study between Human and Canine Species. *Life*. 2021 Dec 1;11(12):1427. DOI: 10.3390/life11121427.
52. Moschetta MG, Leonel C, Maschio-Signorini LB, Borin TF, Gelaleti GB, Jardim-Perassi BV, et al. Evaluation of Angiogenesis Process after Metformin and LY294002 Treatment in Mammary Tumor. *Anti-Cancer Agents in Medicinal Chemistry- Anti-Cancer Agents*. 2019 Mar 1;19(5):655–66. DOI: 10.2174/1871520619666181218164050.

53. Coulson R, Liew SH, Connelly AA, Yee NS, Deb S, Kumar B, et al. The angiotensin receptor blocker, Losartan, inhibits mammary tumor development and progression to invasive carcinoma. *Oncotarget*. 2017 Feb 21;8(12). DOI: 10.18632/oncotarget.15553.
54. Wang J, Yang L, Liang F, Chen Y, Yang G. Integrin alpha x stimulates cancer angiogenesis through PI3K/Akt signaling–mediated VEGFR2/VEGF-A overexpression in blood vessel endothelial cells. *Journal of Cellular Biochemistry*. 2018 Sep 14;120(2):1807–18. DOI: 10.1002/jcb.27480.
55. Khan N, Jajeh F, Eberhardt EL, Miller DD, Albrecht DM, Van Doorn R, et al. Fisetin and 5-fluorouracil: Effective combination for PIK3CA-mutant colorectal cancer. *International Journal of Cancer*. 2019 May 10;145(11):3022–32. DOI: 10.1002/ijc.32367.
56. Varanda AS, Santos M, Soares AR, Vitorino R, Oliveira P, Oliveira C, et al. Human cells adapt to translational errors by modulating protein synthesis rate and protein turnover. *RNA Biology*. 2019 Oct 1;17(1):135–49. DOI: 10.1080/15476286.2019.1670039.

4.5 Material Suplementar Artigo 2

Supplementary Table 1. List of antibodies and tissues used as positive and negative controls.

Primary Antibody	Dilution	Manufacturer	Positive Control*	Negative Control*
Anti-PI3 Kinase Catalytic Subunit Alpha ab135958	1:50	Abcam Mouse monoclonal	Placenta	Liver (bile ducts)
Anti-HIF-1 α sc-53546	1:50	Santa Cruz Biot. Mouse monoclonal	Placenta	Liver
Anti-VHL SAB4200285	1:300	Sigma-Aldrich Rabbit polyclonal	Kidney	Placenta
Anti-Zeb1 HPA027524	1:100	Sigma-Aldrich Rabbit polyclonal	Kidney	Placenta
Anti-Zeb2 SAB2108744	1:500	Sigma-Aldrich Rabbit polyclonal	Kidney	Placenta
Anti-Caspase 3 CP 229	1:50	BioCare Medical Rabbit polyclonal	Colon	Liver
Anti-PARP SI258550	1:50	ThermoFischer Mouse monoclonal	Spleen	Liver
Anti-PTEN SC-7974	1:100	Santa Cruz Biot. Mouse monoclonal	Placenta	Liver

Fonte: *The Human Protein Atlas, 2020.

Supplementary Table 2. Relationship between diagnosis and history of tumors in dogs.

	PC	ES	AC	TE	HISTOPATHOLOGICAL	UPSHOT
T1	No	No	No	> 6 months	Carcinoma tubular grade III	Death
T2	No	No	Yes	> 6 months	Carcinoma tubulopapillary grade I	Death
T3	No	Yes	No	> 6 months	Carcinoma complex type grade I	Live
T4	No	Yes	No	> 6 months	Mammary hemangiosarcoma	Live
T5	No	No	No	> 6 months	Carcinoma tubular grade III	Live
T6	No	No	Yes	> 6 months	Carcinoma tubular grade I	Live
T7	No	No	No	1 month	Carcinoma tubular grade II	Live
T8	No	No	No	> 6 months	Carcinoma anaplastic	Live
T9	No	No	No	> 6 months	Carcinoma tubular grade III	Death
T10	No	No	Yes	> 6 months	Carcinoma complex type grade II	Death
T11	Yes	No	Yes	> 6 months	Carcinoma tubular grade II	Live
T12	No	No	Yes	> 6 months	Carcinoma anaplastic	Death
T13	No	No	No	2 months	Carcinoma tubular grade II	Death

Caption: PC: pseudocyesis; ES: castration; AC: anticio vaccine; TE: evolution time

Supplementary Table 3 – Relationship between clinical and pathological parameters and immunostaining for all patients with breast carcinomas.

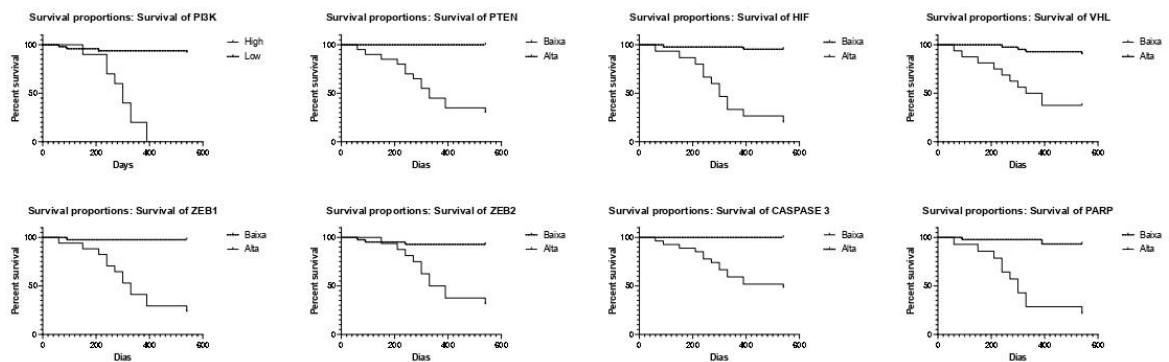
Histopathological	TNM	Phenotype	PI3K	PTEN	HIF1 α	VHL	ZEB1	ZEB2	CASP3	PARP1	Complications	Upshot	Survival Days
Carcinoma in situ	T3N1M1	Her2 +	19	31	45	25	139	3	31	93	Lung metastasis Lung and liver	Death	60
Anaplastic carcinoma	T1N1M1	Her2 +	9	58	20	7	5	4	47	44	metastasis	Death	90
Tubulopapillary carcinoma	T3N1M1	Triple negative	197	11	47	7	154	160	37	68	Lung metastasis	Death	150
Complex carcinoma grade III	T2N0M0	Triple negative	12	5	78	0	105	149	17	64	Lung metastasis	Death	210
Complex carcinoma grade II	-	Triple negative	127	15	119	47	97	39	88	91	Lung metastasis	Death	240
Solid carcinoma grade III	T2N1M0	Luminal B	184	50	136	210	161	140	4	106	Liver metastasis	Death	240
Complex carcinoma grade I	T2N0M0	Triple negative	180	14	87	39	97	159	44	84	Lung metastasis	Death	270
Complex carcinoma grade III	T1N1M0	Luminal B	128	57	94	150	52	174	6	120	Lung metastasis	Death	300
Carcinossarcoma	T3N0M1	Triple negative	249	33	105	21	99	76	65	90	Lung metastasis	Death	300
Tubular carcinoma	T2N1M0	Luminal B	182	23	79	183	131	60	50	164	Lung metastasis	Death	330
Tubulopapillary carcinoma	T3N1M0	Luminal B	164	55	74	31	142	148	7	111	Lung metastasis	Death	330
Complex carcinoma grade III	-	Her2 +	102	43	83	17	139	137	77	25	Lung metastasis	Death	390
Tubulopapillary carcinoma grade II	T1N1M0	Luminal B	153	44	0	26	114	177	38	16	Lung metastasis	Death	390
Complex carcinoma	T1N0M0	Luminal B	18	43	87	153	93	46	153	56	Local recurrence	Live	540
Complex carcinoma	T3N0M0	Luminal B	20	34	3	30	5	0	4	37	No	Live	540
Complex carcinoma grade I	T1N0M0	Her2 +	1	137	1	141	24	22	99	21	No	Live	540
Complex carcinoma grade I	T1N0M0	Luminal A	1	121	0	33	0	0	101	2	No	Live	540
Complex carcinoma grade I	T1N0M0	Luminal B	1	122	0	128	8	107	121	24	No	Live	540
Complex carcinoma grade I	T2N0M0	Luminal A	2	132	2	136	4	45	120	0	No	Live	540
Complex carcinoma grade I	T1N0M0	Luminal B	2	82	25	55	3	33	39	41	No	Live	540
Complex carcinoma grade I	T1N0M0	Luminal B	50	123	3	200	12	0	128	5	No	Live	540
Complex carcinoma grade I	T1N0M0	Luminal A	83	126	0	24	16	11	102	6	No	Live	540
Complex carcinoma grade II	T1N0M0	Luminal B	0	93	1	134	14	4	111	1	No	Live	540
Complex carcinoma grade II	T2N0M0	Luminal A	1	120	0	105	23	38	197	1	No	Live	540
Complex carcinoma grade II	T1N0M0	Luminal A	1	92	5	104	7	5	56	29	No	Live	540

Complex carcinoma grade II	T3N0M0	Luminal A	1	154	0	274	14	101	116	131	No	Live	540
Complex carcinoma grade II	T1N0M0	Luminal A	1	108	24	177	8	56	171	2	No	Live	540
Complex carcinoma grade II	T1N0M0	Luminal B	3	127	1	117	59	1	140	16	No	Live	540
Complex carcinoma grade II	T2N0M0	Luminal B	5	102	2	114	6	41	41	16	No	Live	540
Complex carcinoma grade II	T2N0M0	Luminal B	25	163	16	56	29	20	109	28	No	Live	540
Complex carcinoma grade II	T1N0M0	Luminal A	50	112	19	193	27	53	152	19	No	Live	540
Complex carcinoma grade II	T2N0M0	Luminal B	56	111	0	49	3	1	2	44	No	Live	540
Complex carcinoma grade II	T1N0M0	Luminal A	28	118	9	117	6	53	131	50	No	Live	540
Complex carcinoma grade II	T3N0M0	Luminal A	51	146	16	166	10	1	163	39	No	Live	540
Complex carcinoma grade III	T1N0M0	Luminal A	1	132	0	181	6	1	127	26	No	Live	540
Complex carcinoma grade III	T2N0M0	Luminal A	1	150	0	137	49	0	249	6	No	Live	540
Complex carcinoma grade III	T1N0M0	Luminal A	2	125	1	176	12	54	75	93	No	Live	540
Complex carcinoma grade III	T2N0M0	Triple negative	2	14	0	0	8	6	161	1	No	Live	540
Complex carcinoma grade III	T2N0M0	Luminal B	3	121	56	215	11	68	109	54	No	Live	540
Complex carcinoma grade III	T2N0M0	Luminal B	3	142	1	192	5	31	101	24	No	Live	540
Complex carcinoma grade III	T2N0M0	Luminal B	56	151	0	69	9	60	81	1	No	Live	540
Micropapillary carcinoma	T1N1M0	Luminal A	2	35	0	49	86	0	31	22	Skin metastasis	Live	540
Micropapillary carcinoma	T1N1M0	Luminal B	3	119	0	56	13	1	90	11	No	Live	540
Solid carcinoma grade I	T2N0M0	Luminal A	3	189	0	111	0	4	114	0	No	Live	540
Solid carcinoma grade III	T1N0M1	Luminal A	1	38	124	80	155	187	62	92	Lung metastasis	Death	540
Solid carcinoma grade III	T2N0M0	Luminal B	0	178	0	126	4	11	104	0	No	Live	540
Solid carcinoma grade III	T2N0M0	Luminal A	1	62	0	197	50	0	133	32	No	Live	540
Tubular carcinoma grade II	T3N0M0	Luminal A	2	155	1	65	2	43	133	1	No	Live	540
Tubular carcinoma grade III	T2N1M0	Luminal A	5	127	29	204	17	0	135	2	No	Live	540
Tubulopapillary carcinoma grade I	T2N0M0	Luminal A	92	150	0	85	14	54	213	5	No	Live	540
Tubulopapillary carcinoma grade II	T2N0M0	Luminal B	0	97	1	90	36	26	51	48	Local recurrence	Live	540
Tubulopapillary carcinoma grade II	T2N0M0	Her2 +	1	34	0	214	10	86	75	18	Skin metastasis	Live	540
Tubulopapillary carcinoma grade II	T1N0M0	Luminal B	1	86	64	210	8	20	110	30	No	Live	540

Tubulopapillary carcinoma grade III	T1N0M0	Luminal B	0	173	0	145	2	1	82	34	No	Live	540
Tubulopapillary carcinoma grade III	T3N0M0	Luminal A	4	160	0	172	10	54	102	90	No	Live	540
Tubulopapillary carcinoma grade III	T1N0M0	Luminal A	9	42	0	102	8	2	110	15	No	Live	540
Tubulopapillary carcinoma grade III	T3N0M0	Luminal A	31	167	0	93	1	1	37	36	No	Live	540
Tubulopapillary carcinoma grade III	T1N0M0	Luminal B	37	141	0	63	6	1	69	39	No	Live	540

Supplementary Table 4 – Cut off values and their respective sensitivity and specificity.

Protein	Cut off	Sensitivity (%)	Specificity (%)
PI3K	97	62,5	100
PTEN	60	100	90,5
HIF-1 α	37	75	92,7
VHL	52	68,7	88,1
ZEB1	49,5	92,9	87,5
ZEB2	58	75,0	90,5
CASPASE-3	89	100	73,8
PARP1	60	68,8	92,9

Supplementary Figure 1. Mean overall survival curve for all proteins of positive and negative patients.

Legend: Kaplan-Meier curves comparing the estimated survival, in days, of sick patients with negative and positive labeling, for all $p < 0.0001$.

Supplementary Table 5. Correlation coefficient relationship of the analyzed proteins and PI3K and their p-values.

PI3K vs.	Correlation coefficient (r)	P
PTEN	-0,4634	0,0002
HIF-1 α	0,6062	<0,0001
VHL	-0,2724	0,0386
ZEB1	0,6523	<0,0001
ZEB2	0,5624	<0,0001
CASPASE3	-0,3714	0,0041
PARP1	0,5160	<0,0001

5 CONCLUSÃO

Os resultados finais deste estudo permitem concluir que:

1. A mutação H1047R do gene PIK3CA está presente em cadelas com câncer de mama e parece aumentar a expressão da proteína PI3K e dos alvos desta via ZEB2, HIF1 α e PARP1.
2. As cadelas com alta expressão proteica de PI3K tem associação com metástase a distância e linfonodo regional acometido, bem como diminuição do tempo de sobrevida.
3. A expressão aumentada das proteínas PTEN, HIF-1 α , VHL, ZEB1, ZEB2, CASPASE3 E PARP1 tem correlação, com a alta expressão de PI3K, evidenciando a relação dessas proteínas com a evolução desta neoplasia maligna.
4. Não foi observada associação entre tipo e grau histopatológico com alta expressão proteica de PI3K.
5. A expressão gênica dos genes ZEB2 e PARP1 validada pela imunohistoquímica, confirma o menor tempo de sobrevida da doença.

Sendo assim os resultados confirmam a hipótese prognóstica para estes alvos e estimulam futuros estudos para maior conhecimento das interações e alterações relacionadas à via PI3K/AKT/mTOR e o seu envolvimento na patogênese molecular dos tumores de mama caninos.

REFERÊNCIAS

- ABDELMEGEED, S.; MOHAMMED, S. Canine mammary tumors as a model for human disease (Review). **Oncology Letters**, v. 15, n. 6, 2018.
- ADAMS, J. R. et al. Cooperation between Pik3ca and p53 Mutations in Mouse Mammary Tumor Formation. **Cancer Research**, v. 71, n. 7, p. 2706–2717, 2011.
- ADAMS, R. H.; ALITALO, K. Molecular regulation of angiogenesis and lymphangiogenesis. **Nature Reviews Molecular Cell Biology**, v. 8, n. 6, p. 464–478, 2007.
- ALSAIHATI, B. A. et al. Canine tumor mutational burden is correlated with TP53 mutation across tumor types and breeds. **Nature Communications**, v. 12, n. 1, p. 4670, 2021.
- AMIRKHANI NAMAGERDI, A. et al. Triple-Negative Breast Cancer Comparison With Canine Mammary Tumors From Light Microscopy to Molecular Pathology. **Frontiers in Oncology**, v. 10, p. 563779, 2020.
- AMORNSUPAK, K. et al. High ASMA + Fibroblasts and Low Cytoplasmic HMGB1 + Breast Cancer Cells Predict Poor Prognosis. **Clinical Breast Cancer**, v. 17, n. 6, p. 441-452.e2, 2017.
- APPERT-COLLIN, A. et al. Role of ErbB Receptors in Cancer Cell Migration and Invasion. **Frontiers in Pharmacology**, v. 6, 2015.
- ASPRONI, P. et al. An Immunohistochemical Study of the PTEN/AKT Pathway Involvement in Canine and Feline Mammary Tumors. **Animals**, v. 11, n. 2, p. 365, 2021.
- BACKER, JONATHAN M. The intricate regulation and complex functions of the Class III phosphoinositide 3-kinase Vps34. **Biochemical Journal**, v. 473, n. 15, p. 2251–2271, 2016.
- BARDOU, V.J. et al. Progesterone Receptor Status Significantly Improves Outcome Prediction Over Estrogen Receptor Status Alone for Adjuvant Endocrine Therapy in Two Large Breast Cancer Databases. **Journal of Clinical Oncology**, v. 21, n. 10, p. 1973–1979, 2003.
- BIONDI, L. R. et al. Canine mammary tumors in Santos, Brazil: clinicopathological and survival profile. **Brazilian Journal of Veterinary Research and Animal Science**, v. 51, n. 3, p. 252–262, 16 dez. 2014.
- BLANCHER, C. et al. Relationship of Hypoxia-inducible Factor (HIF)-1 α and HIF-2 α Expression to Vascular Endothelial Growth Factor Induction and Hypoxia Survival in Human Breast Cancer Cell Lines. **Cancer Research**, v. 60, n. 24, p. 7106–7113, 15 dez. 2000.
- BORECKA, P. et al. Expression of Periostin in Cancer-associated Fibroblasts in Mammary Cancer in Female Dogs. **In Vivo**, v. 34, n. 3, p. 1017–1026, 2020.
- BORGE, K. S. et al. Canine Mammary Tumours Are Affected by Frequent Copy

Number Aberrations, including Amplification of MYC and Loss of PTEN. **PLOS ONE**, v. 10, n. 5, 2015.

BRACCINI, L. et al. PI3K-C2 γ is a Rab5 effector selectively controlling endosomal Akt2 activation downstream of insulin signalling. **Nature Communications**, v. 6, n. 1, p. 7400, 2015.

BRUNELLE, J. K. et al. MCL-1–dependent leukemia cells are more sensitive to chemotherapy than BCL-2–dependent counterparts. **Journal of Cell Biology**, v. 187, n. 3, p. 429–442, 2009.

BRUYETTE, D. **Clinical small animal internal medicine**. Chichester: Wiley Blackwell, 2020.

CAIN, R. J.; RIDLEY, A. J. Phosphoinositide 3-kinases in cell migration. **Biology of the Cell**, v. 101, n. 1, p. 13–29, 2009.

CARBOGNIN, L. et al. Prognostic and Predictive Implications of PTEN in Breast Cancer: Unfulfilled Promises but Intriguing Perspectives. **Cancers**, v. 11, n. 9, p. 1401, 2019.

CARMELIET, P.; JAIN, R. K. Angiogenesis in cancer and other diseases. **Nature**, v. 407, n. 6801, p. 249–257, 2000.

CASSALI, G. et al. Consensus Regarding the Diagnosis, Prognosis and Treatment of Canine and Feline Mammary Tumors. **Brazilian Journal of Veterinary Pathology**, v. 13, n. 3, p. 555–574, 2020.

CASSALI, G. et al. Consensus for the diagnosis, prognosis and treatment of canine mammary tumors. **Braz. J. Vet. Pathol.**, v. 4, n. 2, p. 153–180, 2011.

CASSALI, G. D. et al. Consensus for the Diagnosis, Prognosis and Treatment of Canine Mammary Tumors 2013. **Braz. J. Vet. Pathol.**, v. 7, n. 2, p. 38–69, 2014.

CHANG, F. et al. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. **Leukemia**, v. 17, n. 3, p. 590–603, 2003.

CLARHAUT, J. et al. ZEB-1, a Repressor of the Semaphorin 3F Tumor Suppressor Gene in Lung Cancer Cells. **Neoplasia**, v. 11, n. 2, p. 157–IN5, 2009.

CLEMENTE, M. et al. Histological, Immunohistological, and Ultrastructural Description of Vasculogenic Mimicry in Canine Mammary Cancer. **Veterinary Pathology**, v. 47, n. 2, p. 265–274, 2009.

NARDI AB, DALECK CR. **Oncologia de cães e gatos**. Roca, 2016.

COLOMBO, J. et al. Liquid Biopsy as a Diagnostic and Prognostic Tool for Women and Female Dogs with Breast Cancer. **Cancers**, v. 13, n. 20, p. 5233, 2021.

DAI, X. et al. Cancer Hallmarks, Biomarkers and Breast Cancer Molecular Subtypes. **Journal of Cancer**, v. 7, n. 10, p. 1281–1294, 2016.

DERYUGINA, E. I.; QUIGLEY, J. P. Tumor angiogenesis: MMP-mediated induction of intravasation- and metastasis-sustaining neovasculature. **Matrix Biology**, v. 44-46, p. 94–112, 2015.

DICKERSON, E. B. et al. Mutations of Phosphatase and Tensin Homolog Deleted from Chromosome 10 in Canine Hemangiosarcoma. **Veterinary Pathology**, v. 42, n. 5, p. 618–632, 2005.

DONG, L. et al. Cooperative oncogenic effect and cell signaling crosstalk of co-occurring HER2 and mutant PIK3CA in mammary epithelial cells. **International Journal of Oncology**, v. 51, n. 4, p. 1320–1330, 2017.

DONIZY, P. et al. Up-Regulation of PARP1 Expression Significantly Correlated with Poor Survival in Mucosal Melanomas. **Cells**, v. 9, n. 5, p. 1135, 2020.

YANG, QI; JIANG, WEI; HOU, PENG. Emerging role of PI3K/AKT in tumor-related epigenetic regulation. **Seminars in Cancer Biology**, v. 59, p. 112–124, 2019.

FERNALD, K.; KUROKAWA, M. Evading apoptosis in cancer. **Trends in Cell Biology**, v. 23, n. 12, p. 620–633, 2013.

FERREIRA, E. et al. The relationship between tumour size and expression of prognostic markers in benign and malignant canine mammary tumours. **Veterinary and Comparative Oncology**, v. 7, n. 4, p. 230–235, 2009.

GALLYAS, F.; SUMEGI, B.; SZABO, C. Role of Akt Activation in PARP Inhibitor Resistance in Cancer. **Cancers**, v. 12, n. 3, p. 532, 2020.

GAMA, A. A novel myoepithelial cell marker in canine mammary tissue. **Veterinary journal (London, England)**, v. 190, n. 3, p. 303–304, 2011.

GAMA, A.; ALVES, A.; SCHMITT, F. Identification of molecular phenotypes in canine mammary carcinomas with clinical implications: application of the human classification. **Virchows Archiv**, v. 453, n. 2, p. 123–132, 2008.

GOLDSCHMIDT, M. et al. Classification and Grading of Canine Mammary Tumors. **Veterinary Pathology**, v. 48, n. 1, p. 117–131, 2011.

GOSSAGE, L.; EISEN, T.; MAHER, E. R. VHL, the story of a tumour suppressor gene. **Nature Reviews Cancer**, v. 15, n. 1, p. 55–64, 2014.

GRAY, M. et al. Naturally-Occurring Canine Mammary Tumors as a Translational Model for Human Breast Cancer. **Frontiers in Oncology**, v. 10, 2020.

GU, K. et al. Interleukin-17-induced EMT promotes lung cancer cell migration and invasion via NF- κ B/ZEB1 signal pathway. **American Journal of Cancer Research**, v. 5, n. 3, p. 1169–1179, 2015.

HALL, M.; BENAFIF, S. An update on PARP inhibitors for the treatment of cancer. **OncoTargets and Therapy**, n. 8, p. 519, 2015.

HARBECK, N. et al. Breast cancer. **Nature Reviews Disease Primers**, v. 5, n. 1, 2019.

HE, J. et al. Hypoxia-inhibited miR-338-3p suppresses breast cancer progression by directly targeting ZEB2. **Cancer Science**, v. 111, n. 10, p. 3550–3563, 2020.

HUANG, N. et al. MiR-338-3p inhibits epithelial-mesenchymal transition in gastric cancer cells by targeting ZEB2 and MACC1/Met/Akt signaling. **Oncotarget**, v. 6, n. 17, p. 15222–15234, 2015.

JAWHAR, N.M.T. Tissue microarray: a rapidly evolving diagnostic and research tool. **Annals of Saudi medicine**, v. 29, n. 2, p. 123-127, 2009.

JIANG, B.H.; LIU, L.Z. PI3K/PTEN signaling in angiogenesis and tumorigenesis. **Advances in Cancer Research**, v. 102, p. 19–65, 2009.

JURIC, D. et al. Alpelisib Plus Fulvestrant in PIK3CA-Altered and PIK3CA-Wild-Type Estrogen Receptor–Positive Advanced Breast Cancer. **JAMA Oncology**, v. 5, n. 2, 2019.

KANG, K. A. et al. Myricetin Protects Cells against Oxidative Stress-Induced Apoptosis via Regulation of PI3K/Akt and MAPK Signaling Pathways. **International Journal of Molecular Sciences**, v. 11, n. 11, p. 4348–4360, 2010.

KANIECKI, K.; DE TULLIO, L.; GREENE, E. C. A change of view: homologous recombination at single-molecule resolution. **Nature Reviews Genetics**, v. 19, n. 4, p. 191–207, 2018.

KIM, K.-K. et al. Whole-exome and whole-transcriptome sequencing of canine mammary gland tumors. **Scientific Data**, v. 6, n. 1, 2019.

KIM, S.-H. et al. Dysregulation of PI3K/Akt/PTEN Pathway in Canine Mammary Tumor. **Animals**, v. 11, n. 7, p. 2079, 2021.

KIM, T.-M. et al. Cross-species oncogenic signatures of breast cancer in canine mammary tumors. **Nature Communications**, v. 11, n. 1, p. 3616, 2020.

KNAUTH, K. et al. Renal cell carcinoma risk in type 2 von Hippel–Lindau disease correlates with defects in pVHL stability and HIF-1 α interactions. **Oncogene**, v. 25, n. 3, p. 370–377, 2005.

KOENIG, A. et al. Expression and Significance of p53, Rb, p21/waf-1, p16/ink-4a, and PTEN Tumor Suppressors in Canine Melanoma. **Veterinary Pathology**, v. 39, n. 4, p. 458–472, 2002.

KOREN, S. et al. PIK3CAH1047R induces multipotency and multi-lineage mammary

tumours. **Nature**, v. 525, n. 7567, p. 114–118, 2015.

KUMAR, A. et al. Deep feature learning for histopathological image classification of canine mammary tumors and human breast cancer. **Information Sciences**, v. 508, p. 405–421, 2020.

LARSEN, J. E. et al. ZEB1 drives epithelial-to-mesenchymal transition in lung cancer. **The Journal of Clinical Investigation**, v. 126, n. 9, p. 3219–3235, 2016.

LEE, K.-H. et al. Somatic Mutation of PIK3CA (H1047R) Is a Common Driver Mutation Hotspot in Canine Mammary Tumors as Well as Human Breast Cancers. **Cancers**, v. 11, n. 12, p. 2006, 2019.

LI, D. et al. PI3K/Akt and caspase pathways mediate oxidative stress-induced chondrocyte apoptosis. **Cell Stress and Chaperones**, v. 24, n. 1, p. 195–202, 2018.

LI, L. et al. Hypoxia-Inducible Factor Linked to Differential Kidney Cancer Risk Seen with Type 2A and Type 2B VHL Mutations. **Molecular and Cellular Biology**, v. 27, n. 15, p. 5381–5392, 2007.

LI, R. et al. A Novel Canine Mammary Cancer Cell Line: Preliminary Identification and Utilization for Drug Screening Studies. **Frontiers in Veterinary Science**, v. 8, p. 665906, 2021.

LI, W. et al. Retracted: Targeted Regulation of miR-26a on PTEN to Affect Proliferation and Apoptosis of Prostate Cancer Cells. **Cancer Biotherapy and Radiopharmaceuticals**, v. 34, n. 7, p. 480–485, 2019.

LIM, W.; MAYER, B.; T PAWSON. **Cell signaling: principles and mechanisms**. New York: Garland Science, Taylor & Francis Group, 2015.

LIN, J. et al. Genetic Polymorphisms in the Apoptosis-Associated Gene CASP3 and the Risk of Lung Cancer in Chinese Population. **PLOS ONE**, v. 11, n. 10, p. e0164358, 2016.

LIU, Y. et al. Different thresholds of ZEB1 are required for Ras-mediated tumour initiation and metastasis. **Nature Communications**, v. 5, n. 1, 2014.

LUONGO, F. et al. PTEN Tumor-Suppressor: The Dam of Stemness in Cancer. **Cancers**, v. 11, n. 8, p. 1076, 2019.

MADEJ, J. A. et al. Expression of hypoxia-inducible factor-1 α and vascular density in mammary adenomas and adenocarcinomas in bitches. **Acta Veterinaria Scandinavica**, v. 55, n. 1, 2013.

MAHESHWARI, A. et al. N-acetyl-L-cysteine counteracts oxidative stress and prevents H₂O₂ induced germ cell apoptosis through down-regulation of caspase-9 and JNK/c-Jun. **Molecular Reproduction and Development**, v. 78, n. 2, p. 69–79, 2011.

- MANISCALCO, L. et al. Activation of AKT in feline mammary carcinoma: A new prognostic factor for feline mammary tumours. **The Veterinary Journal**, v. 191, n. 1, p. 65–71, 2012.
- MAVRATZAS, A.; MARMÉ, F. Alpelisib in the treatment of metastatic HR+ breast cancer with PIK3CA mutations. **Future Oncology**, 2020.
- MÉNARD, S. et al. Biologic and therapeutic role of HER2 in cancer. **Oncogene**, v. 22, n. 42, p. 6570–6578, 2003.
- MIRICESCU, D. et al. PI3K/AKT/mTOR Signaling Pathway in Breast Cancer: From Molecular Landscape to Clinical Aspects. **International Journal of Molecular Sciences**, v. 22, n. 1, p. 173, 2020.
- MISDORP, W. Histological classification of the mammary tumors of the dog and the cat. **World Health Organization International Histological Classification of Tumors of Domestic Animals, 2nd series**, v. 7, p. 1–59, 1999.
- MOTA, A. D. L. et al. Melatonin modifies tumor hypoxia and metabolism by inhibiting HIF-1 α and energy metabolic pathway in the in vitro and in vivo models of breast cancer. **Melatonin Research**, v. 2, n. 4, p. 83–98, 2019.
- NADERALI, E. et al. Regulation and modulation of PTEN activity. **Molecular Biology Reports**, v. 45, n. 6, p. 2869–2881, 2018.
- OWEN, L. N. **TNM classification of tumors in domestic animals**. 1980
- PAPA, A. et al. Update on Poly-ADP-ribose polymerase inhibition for ovarian cancer treatment. **Journal of Translational Medicine**, v. 14, n. 1, 2016.
- PASTOR, N. et al. Prognostic significance of immunohistochemical markers and histological classification in malignant canine mammary tumours. **Veterinary and Comparative Oncology**, v. 18, n. 4, p. 753–762, 2020.
- PAVAM, M. V. et al. Immunohistochemical evaluation of e-cadherin, Ki-67 and PCNA in canine mammary neoplasias: correlation of prognostic factors and clinical outcome. **Pesquisa Veterinária Brasileira**, 2008.
- PEÑA, L. et al. Canine Mammary Tumors. **Veterinary Pathology**, v. 51, n. 1, p. 127–145, 2013.
- PEREIRA, B. et al. The somatic mutation profiles of 2,433 breast cancers refine their genomic and transcriptomic landscapes. **Nature Communications**, v. 7, n. 1, 2016.
- PORRELLO, A.; CARDELLI, P.; SPUGNINI, E. P. Oncology of companion animals as a model for humans. an overview of tumor histotypes. **Journal of experimental & clinical cancer research: CR**, v. 25, n. 1, p. 97–105, 2006.
- QUEIROGA, F. L. et al. Clinical and prognostic implications of serum and tissue prolactin levels in canine mammary tumours. **Veterinary Record**, v. 175, n. 16, p.

403–403, 2014.

RAMOS-VARA, J. A. Technical aspects of immunohistochemistry. **Veterinary pathology**, v. 42, n. 4, p. 405–26, 2005.

RESSEL, L. et al. Reduced PTEN Protein Expression and Its Prognostic Implications in Canine and Feline Mammary Tumors. **Veterinary Pathology**, v. 46, n. 5, p. 860–868, 2009.

RIVERA-CALDERÓN, L. G. et al. Alterations in PTEN, MDM2, TP53 and AR protein and gene expression are associated with canine prostate carcinogenesis. **Research in Veterinary Science**, v. 106, p. 56–61, 2016.

RUSSELL, D. S.; JAWORSKI, L.; KISSEBERTH, W. C. Immunohistochemical detection of p53, PTEN, Rb, and p16 in canine osteosarcoma using tissue microarray. **Journal of Veterinary Diagnostic Investigation**, v. 30, n. 4, p. 504–509, 2018.

SABA, C. et al. A Comparative Oncology Study of Iniparib Defines Its Pharmacokinetic Profile and Biological Activity in a Naturally-Occurring Canine Cancer Model. **PLOS ONE**, v. 11, n. 2, p. e0149194, 2016.

SHIN, J.I. et al. Analysis of Hypoxia-Inducible Factor-1 α Expression Relative to Other Key Factors in Malignant Canine Mammary Tumours. **Journal of Comparative Pathology**, v. 153, n. 2, p. 101–110, 2015.

SIEMPELKAMP, B. D. et al. Molecular mechanism of activation of class IA phosphoinositide 3-kinases (PI3Ks) by membrane-localized HRas. **Journal of Biological Chemistry**, v. 292, n. 29, p. 12256–12266, 2017.

SINGH, M. et al. EMT: Mechanisms and therapeutic implications. **Pharmacology & Therapeutics**, v. 182, p. 80–94, fev. 2018.

SKINNER, H. D. et al. Vascular Endothelial Growth Factor Transcriptional Activation Is Mediated by Hypoxia-inducible Factor 1 α , HDM2, and p70S6K1 in Response to Phosphatidylinositol 3-Kinase/AKT Signaling. **Journal of Biological Chemistry**, v. 279, n. 44, p. 45643–45651, 2004.

SOBRAL-LEITE, M. et al. Cancer-immune interactions in ER-positive breast cancers: PI3K pathway alterations and tumor-infiltrating lymphocytes. **Breast Cancer Research**, v. 21, n. 1, 2019.

SPOERRI, M. et al. Endocrine control of canine mammary neoplasms: serum reproductive hormone levels and tissue expression of steroid hormone, prolactin and growth hormone receptors. **BMC Veterinary Research**, v. 11, n. 1, 2015.

TANG, Z. et al. Safflower yellow promotes angiogenesis through p-VHL/ HIF-1 α /VEGF signaling pathway in the process of osteogenic differentiation. **Biomedicine & Pharmacotherapy**, v. 107, p. 1736–1743, 2018.

THORPE, L. M. et al. PI3K-p110 α mediates the oncogenic activity induced by loss of the novel tumor suppressor PI3K-p85 α . **Proceedings of the National Academy of**

Sciences, v. 114, n. 27, p. 7095–7100, 2017.

THUMSER-HENNER, P.; NYTKO, K. J.; ROHRER BLEY, C. Mutations of BRCA2 in canine mammary tumors and their targeting potential in clinical therapy. **BMC Veterinary Research**, v. 16, n. 1, 2020.

TIMMERMANS-SPRANG, E. P. M.; GRACANIN, A.; MOL, J. A. Molecular Signaling of Progesterone, Growth Hormone, Wnt, and HER in Mammary Glands of Dogs, Rodents, and Humans: New Treatment Target Identification. **Frontiers in Veterinary Science**, v. 4, 2017.

VARALLO, G. et al. Prognostic phenotypic classification for canine mammary tumors. **Oncology Letters**, 2019.

WANG, G. et al. Canine Oncopanel: A capture-based, NGS platform for evaluating the mutational landscape and detecting putative driver mutations in canine cancers. **Veterinary and Comparative Oncology**, 2021.

WANG, J. et al. Integrin alpha x stimulates cancer angiogenesis through PI3K/Akt signaling–mediated VEGFR2/VEGF-A overexpression in blood vessel endothelial cells. **Journal of Cellular Biochemistry**, v. 120, n. 2, p. 1807–1818, 2018.

WITHROW, S. J.; PAGE, R.; VAIL, D. M. **Withrow and MacEwen's Small Animal Clinical Oncology - E-Book**. [s.l.] Saunders, 2013.

WU, D. et al. The PAX6-ZEB2 axis promotes metastasis and cisplatin resistance in non-small cell lung cancer through PI3K/AKT signaling. **Cell Death & Disease**, v. 10, n. 5, p. 1–15, 2019.

WU, K. et al. PI3K/Akt to GSK3 β / β -catenin signaling cascade coordinates cell colonization for bladder cancer bone metastasis through regulating ZEB1 transcription. **Cellular Signalling**, v. 24, n. 12, p. 2273–2282, 2012.

XIE, Y. et al. PI3K/Akt signaling transduction pathway, erythropoiesis and glycolysis in hypoxia (Review). **Molecular Medicine Reports**, 2018.

YOSHIDA, K. et al. The Relationship between Clinicopathological Features and Expression of Epithelial and Mesenchymal Markers in Spontaneous Canine Mammary Gland Tumors. **Journal of Veterinary Medical Science**, v. 76, n. 10, p. 1321–1327, 2014.

YU, X.; LONG, Y. C.; SHEN, H.-M. Differential regulatory functions of three classes of phosphatidylinositol and phosphoinositide 3-kinases in autophagy. **Autophagy**, v. 11, n. 10, p. 1711–1728, 2015.

YUDUSHKIN, I. Getting the Akt Together: Guiding Intracellular Akt Activity by PI3K. **Biomolecules**, v. 9, n. 2, p. 67, 2019.

YUN, E.-J. . et al. DAB2IP regulates cancer stem cell phenotypes through modulating stem cell factor receptor and ZEB1. **Oncogene**, v. 34, n. 21, p. 2741–2752, 2015.

ZACHARY, J. F.; MCGAVIN, D.; DONALD MCGAVIN. **Bases da Patologia em Veterinária**. [s.l.] Elsevier Editora Ltda, 2018.

ZAHA, D. C. Significance of immunohistochemistry in breast cancer. **World Journal of Clinical Oncology**, v. 5, n. 3, p. 382, 2014.

ZARDAVAS, D. et al. Tumor PIK3CA Genotype and Prognosis in Early-Stage Breast Cancer: A Pooled Analysis of Individual Patient Data. **Journal of Clinical Oncology**, v. 36, n. 10, p. 981, 2018.

ZHANG, G. et al. Long non-coding RNA ZEB2-AS1 promotes the proliferation, metastasis and epithelial mesenchymal transition in triple-negative breast cancer by epigenetically activating ZEB2. **Journal of Cellular and Molecular Medicine**, v. 23, n. 5, p. 3271–3279, 2019.

ZHANG, J.; ZHANG, Q. VHL and Hypoxia Signaling: Beyond HIF in Cancer. **Biomedicines**, v. 6, n. 1, p. 35, 2018.

ZHANG, P.; SUN, Y.; MA, L. ZEB1: At the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance. **Cell Cycle**, v. 14, n. 4, p. 481–487, 2015.

ZHANG, Y. et al. A Pan-Cancer Proteogenomic Atlas of PI3K/AKT/mTOR Pathway Alterations. **Cancer Cell**, v. 31, n. 6, p. 820-832.e3, 2017.

ZHANG, Y. et al. The roles of ZEB1 in tumorigenic progression and epigenetic modifications. **Biomedicine & Pharmacotherapy**, v. 110, p. 400–408, 2019.

ZHENG, X. et al. Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. **Nature**, v. 527, n. 7579, p. 525–530, 2015.

ZUSAK, M. A menina que roubava livros. 1ª ed. Brasil: Intrínseca, 2007.