CECILIA BONOLO DE CAMPOS

AVALIAÇÃO DE FATORES PROGNÓSTICOS E TRATAMENTO QUIMIOTERÁPICO ADJUVANTE EM NEOPLASIAS MAMÁRIAS MALIGNAS FELINAS

Belo Horizonte

Fevereiro de 2013

UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA DA UFMG

FACULDADE DE MEDICINA

DISSERTAÇÃO DE MESTRADO

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Dissertação apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina da Universidade Federal de Minas Gerais como parte dos requisitos para a obtenção do título de mestre.

Área de Concentração: Patologia Investigativa

Orientador: Prof. Dr. Geovanni Dantas Cassali

Co-orientadora: Dra. Gleidice Eunice Lavalle

Belo Horizonte

Faculdade de Medicina - UFMG

2013

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ATA DA DEFESA DE DISSERTAÇÃO DE MESTRADO de **CECÍLIA BONOLO DE CAMPOS**, nº de registro 2011655808. Às treze horas do dia **25 de fevereiro de dois mil e treze**, reuniu-se no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora de defesa de dissertação, indicada pelo Colegiado do Programa de Pós-Graduação em Patologia da UFMG, para julgar, em exame final, o trabalho intitulado: **"AVALIAÇÃO DE FATORES PROGNÓSTICOS E TRATAMENTO QUIMIOTERÁPICO ADJUVANTE EM NEOPLASIAS MAMÁRIAS MALIGNAS FELINAS"**, requisito final para a obtenção do grau de Mestre em Patologia, pelo Programa de Pós-Graduação em Patologia da UFMG - Área de Concentração em Patologia Investigativa. Abrindo a sessão, o Presidente da Comissão, Prof. Geovanni Dantas Cassali, após dar a conhecer aos presentes o teor das normas regulamentares do trabalho final passou a palavra à candidata para apresentação do seu trabalho. Seguiu-se a arguição pelos examinadores com a respectiva defesa da candidata. Logo após, a Comissão se reuniu sem a presença da candidata e do público para julgamento e expedição do resultado final. Foram atribuídas as seguintes indicações:

Prof. Geovanni Dantas Cassali/Orientador	Instituição: UFMG	Indicação: Aprovado
Dra. Gleidice Eunice Lavalle/Coorientadora	Instituição: UFMG	Indicação: Aprovado
Profa. Renee Laufer Amorim	Instituição: UNESP	Indicação: aprovado
Prof. Carlos Roberto Daleck	Instituição: UNESP	Indicação: Aprovado

Pelas indicações, a candidata foi considerada Aprovada.

O resultado final foi comunicado publicamente à candidata pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. Belo Horizonte, 25 de fevereiro de 2013.

Prof. Geovanni Dantas Cassali _____

Dra. Gleidice Eunice Lavalle _____

Profa. Renee Laufer Amorim _____

Prof. Carlos Roberto Daleck _____

Profa. Rosa Maria Esteves Arantes (Coordenadora) _____

Obs.: Este documento não terá validade sem a assinatura e carimbo do Coordenador.

Profa. Rosa Maria Esteve Arantes
Coordenadora do Programa de
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DECLARAÇÃO

A Comissão Examinadora, abaixo assinada, composta pelos professores doutores: Geovanni Dantas Cassali, Gleidice Eunice Lavalle, Renee Laufer Amorim e Carlos Roberto Daleck, aprovou a defesa da dissertação intitulada: **"AVALIAÇÃO DE FATORES PROGNÓSTICOS E TRATAMENTO QUIMIOTERÁPICO ADJUVANTE EM NEOPLASIAS MAMÁRIAS MALIGNAS FELINAS"**, apresentada pela mestranda **CECÍLIA BONOLO DE CAMPOS**, para obtenção do título de Mestre em Patologia, pelo Programa de Pós-Graduação em Patologia - Área de Concentração em Patologia Investigativa, da Universidade Federal de Minas Gerais, realizada em 25 de fevereiro de 2013.

Prof. Geovanni Dantas Cassali
Orientador

Profa. Gleidice Eunice Lavalle
Coorientadora

Prof. Renee Laufer Amorim

Prof. Carlos Roberto Daleck

Profa. Rosa Maria Esteve Arantes
Coordenadora do Programa de
Pós-Graduaçao em Patologia
Faculdade de Medicina / UFMG

DEDICATÓRIA

Lembro de, ainda criança, ver a tese do meu pai na estante de casa. Diziam que ele havia escrito um livro, e por mais que não tinha desenhos ou sequer parecia interessante, ficava orgulhosa. Já no início da adolescência, minha mãe entrou no doutorado. Disse que a pós-graduação era um momento que estudaria muito para descobrir algo inédito no mundo. Assim, muito antes da graduação, já desejava escrever um livro com descobertas inéditas...

Aos meus pais, Palmira e Francisco.

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“The nontoxic curative compound remains undiscovered

But not undreamt”

James F. Holland

Este trabalho foi realizado no Laboratório de Patologia Comparada do Departamento de Patologia Geral – ICB / UFMG, com apoio financeiro do CNPq, FAPEMIG e CAPES.

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AM – Adenomioepitelioma Maligno

ASCO/CAP - Sociedade Americana de Oncologia Clínica/Colégio Americano de Patologia / American Society of Clinical Oncology/College of American Pathologists

CETEA – Comitê de Ética em Experimentação Animal / Ethics Committee for Animal Experimentation

CGA – Campo de Grande Aumento

Cox-2 – Ciclooxigenase-2 / Cyclooxigenase-2

CRG – Carcinoma Rico em Glicogênio

DAB - 3,3-Diaminobenzidina / 3,3-Diaminobenzidine

DNA – Ácido Desoxirribonucleico

ER – Estrogen Receptor

ET – Endocrine Therapies

FMCs – Feline Mammary Carcinomas

GRCCC – Glycogen-rich Clear Cell Carcinoma

HE – Hematoxilina-Eosina / Hematoxylin and Eosin

Her-2 - Receptor de Fator de Crescimento Epidermal tipo 2 / Human Epidermal Growth Factor Receptor type 2

MA – Malignant Adenomyoepithelioma

MFMGN – Malignant Feline Mammary Gland Neoplasm

OMS – Organização Mundial da Saúde

OS – Overall Survival

PAS – Ácido Periódico de Schiff / Periodic Acid-Schiff

PR – Progesterone Receptor

RE – Receptor de Estrógeno

RP – Receptor de Progesterona

SG – Sobrevida Global

UFMG – Universidade Federal de Minas Gerais / Federal University of Minas Gerais

VEGF – Fator de Crescimento do Endotélio Vascular / Vascular Endothelial Growth Factor

WHO – World Health Organization

RESUMO

As neoplasias mamárias são o terceiro tipo mais frequente de tumores em felinos. O tempo de sobrevida médio é considerado inferior a um ano e prognóstico reservado a desfavorável. A cirurgia é o principal tratamento preconizado para tumores mamários felinos. Estudos clínicos adicionais são necessários para avaliar protocolos quimioterápicos mais eficientes em aumentar o tempo de sobrevida global (SG). Além disso, o conhecimento de fatores prognósticos ou preditivos associado ao comportamento biológico das doenças neoplásicas permite estratégias terapêuticas individualizadas que permitem um aumento no tempo livre de doença e na SG. Sendo assim, o objetivo desse trabalho foi avaliar a conduta terapêutica cirúrgica associada ou não com quimioterapia com carboplatina, comparando ambos os tratamentos por meio da SG; investigar a associação de fatores prognósticos sugeridos na literatura para neoplasias mamárias felinas, associando com a SG; além de descrever, pela primeira vez na espécie felina, as características histopatológicas, imuno-histoquímicas, e clínicas de um adenomioepitelioma maligno (AM) e carcinoma rico em glicogênio (CRG). Para a avaliação terapêutica foram estudadas 16 gatas, divididas em um grupo tratado apenas com cirurgia e outro tratado com cirurgia associado com carboplatina. Para a avaliação dos fatores prognósticos, 37 gatas portadoras de neoplasias malignas foram avaliadas com relação ao seu estadiamento clínico, grau histológico, tipo de diagnóstico e perfil imuno-histoquímico para Ki-67, RE, RP, Her-2, Cox-2 e VEGF, correlacionando os achados com a SG. Não foi observada diferença estatisticamente significativa na SG com relação aos diferentes tratamentos analisados (p=0,883). Com relação aos fatores prognósticos, 35% apresentava metástase, a maioria foi classificada como estadio III, como carcinomas túbulo-papilares e como grau II. Prevaleceu carcinomas mamários positivos para receptores hormonais, Her-2 negativos e com superexpressão de VEGF. A imunoreatividade para Ki-67 (p=0,046) e Cox-2 (p=0,007) foi maior nas metástases em relação aos tumores primários. A expressão proteica de Cox-2 (p=0,089), Her-2 (p=0,012) e grau histológico (p=0,080) foram correlacionados com a SG. A coloração histoquímica com PAS para CGR e imuno-histoquímica com p63 para AM auxiliou na finalização dos diagnósticos. Ambas as neoplasias apresentaram comportamento biológico agressivo, apresentando metástase em linfonodo regional. Os resultados sugerem que o benefício da carboplatina permanece invalidado para carcinomas mamários felinos, e que possivelmente uma inibição de hormônios ovarianos e Cox-2 podem

representar uma opção terapêutica. Além disso, Cox-2 e Ki-67 devem ser avaliados em tumores primários e linfonodos, e que grau histológico, status de Her-2 e Cox-2 influenciam diretamente na SG de felinos com neoplasia mamárias malignas.

ABSTRACT

Mammary neoplasms are the third most frequent tumor in felines. Median overall survival is considered as less than one year and prognosis is guarded to poor. Surgery is the main treatment option for feline mammary neoplasms. Additional clinical studies are necessary in order to evaluate more efficient chemotherapy protocols that may increase overall survival (OS). Furthermore, the understanding of prognostic and predictive factors associated to the biological behavior of neoplastic diseases enables individualized therapeutic strategies aiming towards an increase in disease free interval and overall survival (OS). Therefore, the aim of this study was to evaluate the surgical therapeutic strategy associated or not to chemotherapy with carboplatin, comparing both treatments through OS; investigate the association of prognostic factors suggested by the literature for feline mammary neoplasms, associating data to OS; in addition, to describe, for the first time in the feline species, histopathological, immunohistochemical, and clinical of a malignant adenomyoepithelioma (MA) and glycogen-rich clear cell carcinoma (GRCCC). For the therapeutic evaluation, 16 queens were studied, divided into a group only treated with surgery and another treated with surgery associated to carboplatin. For the evaluation of prognostic factors, 37 queens diagnosed with malignant mammary neoplasms were evaluated. Clinical staging, histological type and grade, and immunohistochemical profile for Ki-67, ER, PR, Her-2, Cox-2, and VEGF were correlated to OS findings. No statistical significant difference in OS was observed when comparing the different treatments (p=0.883). Regarding the prognostic factors, 35% presented metastasis; the majority was classified as stage III, tubulopapillary carcinomas and grade II. Mammary carcinomas positive for hormonal receptors, negative for Her-2, and with superexpression of VEGF prevailed. Immunolabeling for Ki-67 (p=0.046) and Cox-2 (p=0.007) were higher in metastases than in primary tumors. Protein expression of Cox-2 (p=0.089), Her-2 (p=0.012) and histological grade (p=0.080) were correlated to OS. Diagnosis was confirmed through PAS staining for GRCCC and immunohistochemical analysis of p63 for the MA. Both neoplasms presented aggressive biological behavior, characterized by lymph node metastasis. Results suggest that the therapeutic benefit of carboplatin remains invalidated for feline mammary carcinomas, and that the inhibition of ovarian hormones and Cox-2 may possibly represent a therapeutic option. Furthermore, Cox-2 and Ki-67 should be evaluated in primary tumors and lymph nodes, and that histological grade, Her-2, and Cox-2 directly influence in OS.

1. INTRODUÇÃO

A longevidade dos animais de companhia está aumentando (WITHROW, 2007), levando a um maior número de animais com risco de desenvolvimento de câncer. Neste cenário, a necessidade de se conhecer melhor os fatores prognósticos e sua aplicação na clínica veterinária aumentam a partir da conscientização da necessidade de cuidados na saúde desses animais associado a uma melhora dos serviços clínicos veterinários (MATOS *et al*., 2012).

2. REVISÃO DE LITERATURA

2.1. Neoplasias mamárias felinas: Aspectos clínicos e patológicos

As neoplasias mamárias são o terceiro tipo mais frequente de tumores em felinos, depois de neoplasias hematopoiéticas e cutâneas (MACEWEN *et al*., 1984; MISDORP, 2002; OVERLEY *et al*., 2005; LANA *et al*., 2007). Sua maior incidência é observada entre 10 e 11 anos de idade (MISDORP, 2002) e em fêmeas não castradas (LANA *et al*., 2007).

Em contraste com humanos e caninos, pelo menos 80% dos tumores mamários felinos são malignos (BOSTOCK, 1986; MISDORP *et al*., 1999). Muitos destes, principalmente tumores grandes e mais invasivos, aderem à pele e são ulcerados. Além disso, a invasão de vasos linfáticos e linfonodos é comum. Metástases regionais ou à distância podem ser encontradas em mais de 80% de gatos com neoplasias mamárias malignas (LANA *et al*., 2007). O prognóstico é considerado reservado a desfavorável e o tempo de sobrevida média inferior a um ano (OVERLEY *et al*., 2005; LANA *et al*,. 2007).

2.2. Neoplasias mamárias felinas: Tratamento

A cirurgia é o principal tratamento preconizado para tumores mamários felinos (MISDORP, 2002) e pode ser realizada em combinação com quimioterapia ou outros tipos de terapias (LANA *et al*., 2007). A mastectomia radical unilateral ou bilateral, independente do tamanho tumoral, é o método cirúrgico de escolha pois reduz a recorrência tumoral, apesar de não promover diferença na sobrevida global (SG) no paciente (MACEWEN *et al.*, 1984; LANA *et al.*, 2007; MCNEILL *et al.*, 2009). Apenas um estudo encontrou uma diferença significativa de SG associada ao tipo de procedimento cirúrgico, gatas submetidas a mastectomias radicais bilaterais tiveram maiores sobrevidas (NOVOSAD, *et al.*, 2006).

A resposta à quimioterapia pode ser insuficiente após a ocorrência de metástases (GIMENÉZ *et al.*, 2010). Os protocolos quimioterápicos propostos pela literatura consistem do uso de doxorubicina como fármaco único ou em associação à ciclofosfamida, carboplatina como fármaco único ou em associação com a doxorubicina e associação de mitoxantrona e ciclofosfamida (KITCHELL *et al*., 1999; LANA *et al*., 2007). Estudos clínicos adicionais devem ser conduzidos para avaliar quais doses e combinações quimioterápicas são mais eficientes em aumentar o tempo de SG em gatas com neoplasias mamárias malignas (GIMENÉZ *et al*., 2010).

Estudos prévios avaliaram o uso da doxorubicina como quimioterapia adjuvante em neoplasias mamárias malignas felinas (NOVOSAD *et al.*, 2006; MCNEILL *et al.*, 2009) e a associação desse quimioterápico com inibidores de Cox-2 (BORREGO *et al.*, 2009). Novosad *et al.* (2006) relataram um aumento na sobrevida ao tratamento de cirurgia associado à doxorubicina (mediana de 641 dias após cinco ciclos de quimioterapia), porém o estudo não incluiu uma população controle. Borrego *et al.* (2009) encontraram uma mediana de SG também alta (mediana de 460 dias) ao tratar felinos portadores de neoplasias mamárias malignas com cirurgia, doxorubicina e inibidor de Cox-2; porém, esse estudo também não incluiu uma população controle. McNeill *et al.* (2009) não observaram efeito benéfico da associação da quimioterapia à cirurgia (mediana 848 dias) quando comparado à gatas tratadas apenas com cirurgia (mediana 1406 dias). Além disso, a doxorubicina pode ser nefrotóxica em felinos necessitando de avaliação cuidadosa da função renal (LANA *et al*., 2007).

O quimioterápico carboplatina é uma medicação antineoplásica da classe dos agentes mistos, reage com o DNA celular formando uniões cruzadas intracelulares e intercelulares e atua em qualquer fase do ciclo celular. Os principais efeitos colaterais são: mielosupressão, alopecia e toxicidade gastrointestinal, podendo ocorrer ainda neuropatias, nefropatias e raramente emêse (ALMEIDA, 2004).

A carboplatina é indicada para o tratamento de neoplasias malignas da glândula mamária de cães e gatos (KITCHELL *et al.*, 1999). A quimioterapia adjuvante com carboplatina foi demonstrada como benéfica no tratamento de cadelas com neoplasias malignas mamárias em estadiamento clínico avançado (LAVALLE *et al.*, 2012). O fármaco também foi proposto como agente de terapia de resgate como único fármaco ou em associação com outros

quimioterápicos (mitoxantrona e doxorubicina) para neoplasias mamárias malignas felinas apresentando recorrências ou metástases (NOVOSAD *et al.*, 2006).

2.3. Neoplasias mamárias felinas: Fatores prognósticos

O conhecimento dos fatores prognósticos é de fundamental importância na determinação dos programas terapêuticos para pacientes humanos com câncer de mama, com intensidade e efetividades adequadas e individualizadas (ABREU; KOIFMAN, 2002). Fatores prognósticos são características clínicas, patológicas e biológicas dos indivíduos e seus tumores que permitem prever a evolução clínica e a sobrevida do paciente (ALLRED *et al.*, 1998). O tamanho tumoral é considerado um importante fator prognóstico em gatas (MACEWEN *et al.*, 1984; FOX *et al.*, 1995; MACY, 1997; VISTE *et al.*, 2002; OVERLEY *et al.*, 2005; LANA *et al.*, 2007; BORREGO *et al.*, 2009), interferindo na SG e sobrevida livre de doença. Outros fatores são extensão da cirurgia, tipo e grau histológico, índice mitótico, estadiamento clínico, comprometimento de linfonodos regionais e metástase à distância (GIMENÉZ *et al.*, 2010; NOVOSAD *et al.*, 2006; HUGHES; DOBSON, 2012; LANA *et al.*, 2007)

Além disso, diversos marcadores moleculares tumorais também são utilizados em tumores mamários felinos, incluindo: os receptores hormonais (estrógeno e progesterona), receptores de fatores de crescimento epidermal (Her-2), ciclooxigenase-2 (COX-2), fator de crescimento do endotélio vascular (VEGF), e os marcadores de proliferação celular (Ki-67) (GIMENÉZ *et al.*, 2010; HUGHES; DOBSON, 2012).

O estadiamento clínico leva em conta a avaliação do tumor primário, linfonodos regionais e possíveis sítios de metástases à distância (LANA *et al.*, 2007). Borrego *et al.* (2009) não demonstraram valor prognósticodao estadiamento clínico pois não foi observado diferença entre medianas de sobrevida livre de doença e SG. Em contrapartida, Novosad *et al.* (2006) demonstraram sobrevida livre de doença significativamente mais longa para animais classificados como estadio I.

Em carcinomas de mama humano, a classificação histológica pode fornecer importantes informações prognósticas (ELLIS *et al.* 1992). Em carcinomas mamários felinos, Seixas *et al.* (2011) observaram maior SG associada a carcinomas túbulo-papilares e complexos e menor SG em carcinomas sólidos e micropapilares. Novosad *et al.* (2006) descreveram menor SG associada a carcinomas anaplásicos e maior SG a carcinomas papilares e tubulares.

No câncer de mama humano, o grau histológico demonstra uma correlação forte com prognostico (ELSTON; ELLIS, 1991). O método Elston e Ellis foi descrito como confiável, sendo um fator prognóstico independente em neoplasias mamárias malignas em gatas (SEIXAS *et al*., 2011). Seu valor prognóstico foi demonstrado com uma SG mais curta associada com tumores mais indiferenciados (SEIXAS *et al*., 2011; MILLANTA *et al*., 2002a).

O índice de marcação imuno-histoquímica de Ki-67 é um excelente marcador para determinar a fração de crescimento em uma determinada população (MILLANTA *et al*., 2002a). Ao usar um ponto de corte de 10% de marcação de Ki-67, o câncer de mama em mulheres pode ser dividido em dois subgrupos que apresentam diferenças estatisticamente significativas entre sobrevida específica do câncer de mama e sobrevida livre de metástase (ALESKANDARANY *et al.*, 2011). Estudos prévios em lesões mamárias em felinos (MILLANTA *et al*., 2002a; DIAS PEREIRA *et al.* 2004; RASOTTO *et al*., 2011) descrevem um aumento progressivo no índice proliferativo a partir da avaliação de mama normal, lesões não neoplásicas, neoplasias benignas, carcinomas *in situ* e carcinomas invasores. Apesar disso, Millanta *et al.* (2002a) não observaram associação entre o índice de Ki-67 e SG em gatas.

O papel dos hormônios ovarianos em neoplasias mamárias felinas foi evidenciado por Overley *et al.* (2005) e Hayden *et al.* (1989), por verificar perda da proteção da doença com o aumento de exposição hormonal, prevalência de gatas inteiras com tumores mamários quando comparados à população castrada e ocorrência esporádica de lesões mamárias concomitantes ao uso de progestágenos sintéticos. Apesar disso, as neoplasias mamárias felinas são consideradas modelos de câncer de mama hormônio-independente na mulher, devido à maioria dos tumores ser considerado negativo para RE e RP (LAS MULAS *et al*., 2000; LAS MULAS *et al*., 2002; MILLANTA *et al*., 2005a; LANA *et al*., 2007). Millanta *et al.* (2005a) não observaram associação entre a presença de expressão de RE e RP e SG em neoplasias mamárias felinas.

Em oncologia humana, a amplificação do gene *HER-2* e / ou a superexpressão de seu receptor estão ligados a intervalos livres de doença mais curtos, aumento no risco de metástase e resistência a muitos tipos de terapias (MILLANTA *et al.*, 2005b). Em gatas, Ordás *et al.* (2007) descreveram uma associação entre tumores apresentando superexpressão proteica de

Her-2 e características indicativas de maior malignidade: tamanho maior, alto grau histológico e ausência de receptores hormonais. Além disso, a importância prognóstica da expressão de Her-2 foi demonstrado por Millanta *et al.* (2005b) por associar diferenças na SG com diferentes escores de Her-2.

A Cox-2 é uma enzima rapidamente induzida, envolvida no processo de transformação maligna e progressão tumoral por afetar a proliferação e adesão celular, apoptose, vigilância imunológica e angiogênese (WILLIAMS *et al.*, 1999; MILLANTA *et al*., 2006). Expressão elevada de Cox-2 foi associado com pior prognóstico, demonstrado por diferenças na SG, descrito por Millanta *et al*. (2006).

O VEGF é um potente fator angiogênico envolvido no crescimento tumoral, invasão e metástase (MILLANTA *et al*., 2002b; GIMENEZ *et al*., 2010; TSAI *et al*., 2012). Em tumores mamários felinos, Millanta *et al*. (2002b) encontraram expressão de VEGF em todos os carcinomas estudados e houve correlação com prognóstico e desfecho clínico.

3. JUSTIFICATIVA

O conhecimento de fatores prognósticos associado ao comportamento biológico de doenças neoplásicas permite estratégias terapêuticas individualizadas, adequadas para cada paciente. A implantação de tratamentos mais eficientes torna-se necessário na tentativa de aumentar a sobrevida de pacientes com tumores mamários felinos. Assim, há uma necessidade em se estudar fatores prognósticos, além de avaliar a eficácia de possíveis tratamentos adjuvantes em felinos portadores de neoplasias mamárias.

4. HIPÓTESE

O tratamento com quimioterapia adjuvante com carboplatina associado ao tratamento cirúrgico proporciona um aumento de sobrevida global ao paciente felino.

Fatores prognósticos já descritos na literatura para tumores malignos possuem associação entre si e com a sobrevida global do paciente felino.

5. OBJETIVOS

5.1. Objetivo geral

Avaliar a associação de fatores prognósticos descritos pela literatura para neoplasias malignas da glândula mamária em felinos, assim como a eficácia da utilização de quimioterapia com carboplatina na terapêutica da doença, comparando os dados obtidos com a sobrevida global.

5.2. Objetivos específicos

- Verificar o benefício terapêutico da quimioterapia adjuvante com carboplatina associado à cirurgia comparado com o tratamento cirúrgico isolado em gatas portadoras de carcinomas mamários, por meio da avaliação da sobrevida global;

- Caracterizar as neoplasias malignas da glândula mamária de felinos quanto ao: tamanho tumoral, presença de ulceração, estadiamento clínico (TNM), aspectos histopatológicos, graduação histológica, e expressão immuno-histoquímica de Ki-67, RE, RP, Cox-2, Her-2 e VEGF, correlacionando esses dados entre si e com a sobrevida global;

- Comparar os dados dos fatores prognósticos imuno-hitoquímicos obtidos entre os tumores primários e metástases.

6. MATERIAL E MÉTODOS

6.1. Aspectos éticos

O projeto foi aprovado pelo Comitê de Ética em Experimentação Animal (CETEA/UFMG) sob protocolo nº 13412/2012 (Anexo A).

6.2. Seleção dos Animais

6.2.1. Para avaliação dos fatores prognósticos em tumores mamários felinos, foram selecionadas 37 gatas portadoras de neoplasias mamárias malignas com histórico clínico, obtidos de material de arquivo do setor de Patologia do Departamento de Clínica e Cirurgias Veterinárias da Escola de Veterinária e do Laboratório de Patologia Comparada do Instituto de Ciências Biológicas da UFMG.

6.2.2. Para avaliar o efeito terapêutico da quimioterapia adjuvante, animais provenientes do Hospital Veterinário da Escola de Veterinária da UFMG foram divididos em dois grupos:

Grupo 1 – Animais portadores de carcinomas mamários submetidos ao tratamento cirúrgico isolado. Estudo retrospectivo.

Grupo 2 - Animais portadores de carcinomas mamários submetidos à exérese cirúrgica convencional e à medicação com quatro ciclos de carboplatina na dosagem de 200mg/m^2, com intervalo de 21 dias. Estudo retrospectivo.

6.3. Obtenção de dados clínicos

O acompanhamento dos animais foi obtido através da avaliação das fichas clínicas dos animais e entrevistas por meio de telefone. Foram avaliados: a evolução clínica das neoplasias mamárias com possíveis recorrências e metástases, outras doenças concomitantes, SG e causa do óbito do animal.

6.4. Estadiamento Clínico

O estadiamento clínico foi realizado de acordo com um sistema de estadiamento clínico modificado da Organização Mundial da Saúde para tumores de mama em felinos. Esse sistema avalia o tamanho do tumor (T_1: 0-2 cm; T_2: 2-3 cm; T_3: >3 cm), o envolvimento de linfonodos regionais (N_0: sem metástase; N_1: metástase) e a presença de metástase à distância (M_0: sem metástase; M_1: metástase). Em seguida, os casos são divididos em quatro estadios: I ($T_1N_0M_0$), II ($T_2N_0M_0$), III ($T_3N_{0\text{-}1}M_0$, $T_{1\text{-}2}N_1M_0$), e IV ($T_{1,2,3}N_{0\text{-}1}M_1$) (BORREGO *et al.*, 2009; MCNEILL *et al.*, 2009).

6.5. Histopatologia

Foram obtidas secções histológicas de 4 μm da glândula mamária, fixados por 48 horas em formol neutro e tamponado a 10%, processados pela técnica rotineira de inclusão em parafina e coradas pela técnica de hematoxilina-eosina (HE). Os tumores foram classificados de acordo com os critérios histológicos veterinários estabelecidos pela OMS (MISDORP *et al*., 1999) e tumores que possuíam múltiplos padrões morfológicos foram classificados de acordo com o padrão predominante na neoplasia.

6.6. Graduação Histológica

Os tumores foram graduados pelo Sistema de Nottingham (ELSTON; ELLIS, 1998), e os critérios incluíram formação tubular, pleomorfismo nuclear e contagem mitótica. As

estruturas tubulares foram definidas como aquelas que apresentaram lúmen claro e visível. O escore 1 foi atribuído aos tumores que apresentarem mais de 75% da área carcinomatosa formada por túbulos; escore 2 entre 10-75% e escore 3 entre 0-10% da área do tumor. Para análise de pleomorfismo nuclear, o tamanho e forma dos núcleos das células epiteliais normais adjacentes ao tumor, foram avaliados como parâmetro. Escore 1 foi atribuído para núcleos pequenos e regulares e cromatina uniforme. Quando os núcleos apresentaram-se de tamanho aumentado e com variabilidade foi dado escore 2. A presença de células com acentuado pleomorfismo, com grande variação no tamanho e forma do núcleo, apresentando ainda núcleos bizarros e vesiculosos, com múltiplos nucléolos caracterizaram o escore 3. Foi utilizado o microscópio BX-41 com ocular de 10x e objetiva de 40x. Este equipamento fornece uma área de 0,237 mm visualizada em campo de grande aumento (CGA). As figuras mitóticas foram contadas em 10 CGA, selecionados preferencialmente na periferia do tumor onde se observa maior atividade de proliferação celular (ELSTON; ELLIS, 1991; ELSTON; ELLIS, 1998). O escore foi atribuído de acordo com o número de mitoses detectadas: escore 1 (0-8 mitoses); escore 2 (9-16 mitoses) e escore 3 (acima de 17 mitoses). Núcleos picnóticos ou hipercromáticos não foram contados uma vez que estas células poderiam estar relacionadas com processos de necrose ou apoptose.

Para a obtenção do grau histológico combinado do tumor, o escore para cada fator foi somado, resultando em um valor total que varia de 3 a 9. O grau tumoral foi alocado baseado nos valores a seguir: 3 – 5 pontos: grau I – baixo grau; 6 – 7 pontos: grau II – grau intermediário; 8 – 9 pontos: grau III – alto grau.

6.7. Imuno-histoquímica

Após a revisão dos casos, secções histológicas de 4 μm das amostras selecionadas foram utilizadas para a técnica de imuno-histoquímica. Detalhes relacionados com anticorpo, clone, fonte, diluição, método de recuperação antigênica e tempo de incubação estão descritos na tabela 1. O procedimento imuno-histoquímico foi identificado a partir de anticorpo secundário polimerizado (ADVANCE HRP – ready to use – DakoCytomation). Para realização de bloqueio da peroxidase endógena as lâminas foram incubadas em solução de H_20_2 3% em álcool metílico. Os reagentes foram aplicados pela técnica manual, sendo o tempo de incubação dos reagentes de 30 minutos, com exceção do cromógeno DAB (DAB substrate system, Dakocytomation), de 5 minutos. Como controles positivos foram usados amostras de

glândulas mamárias felinas previamente testadas para Ki-67, RE, RP e Her-2; secções de carcinoma de cólon humano sabidamente positivo para Cox-2, secções de carcinoma mamário humano sabidamente positivo para VEGF e amostras de mama normal para p63 foram usados como controle positivos. Os controles negativos foram obtidos por substituição do anticorpo primário pelo soro normal (Ultra V Block, Laboratory Vision).

O índice de proliferação foi calculado pela contagem de núcleos positivos para Ki67 em um total de 1000 células neoplásicas (aumento de 400x) (DUTRA *et al*., 2004). Casos foram considerados positivos para RE e RP quando a marcação nuclear estava presente em pelo menos 1% de células tumorais (HAMMOND *et al.*, 2010). A expressão de Her-2 foi determinada pela marcação membranar em células epiteliais e o escore foi determinado de acordo com a Sociedade Americana de Oncologia Clínica/Colégio Americano de Patologia (ASCO/CAP). Amostras foram classificadas em: negativo com escore 0 ou 1+, indeterminado com escore 2+ e positivo com escore 3+. Escore 0 foi caracterizado por marcação membranar ausente; 1+ fraca, incompleta marcação membranar em qualquer proporção de células tumorais; 2+ marcação membranar completa não uniforme ou de intensidade fraca porém com óbvia distribuição circunferencial em pelo menos 10% das células, ou marcação intensa e uniforme em 30% ou menos células tumorais; 3+ completa, uniforme e intensa marcação membranar de mais de 30% das células tumorais. Superexpressão de Her-2 foi considerado apenas em amostras escore 3+ (WOLFF *et al.*, 2007). Positividade para Cox-2 foi indicada pela presença de marcação citoplasmática. O número de células positivas para Cox-2 foi avaliado de forma semi-quantitativa com o escore de distribuição definido pela estimativa de porcentagem de células positivas em cinco campos de 400X. Para todos os tumores foi dado um valor para distribuição de: 0, caracterizado por ausência de marcação; 1, menos de 10% de células marcadas, 2, entre 10 e 30% de marcação; 3, entre 31 e 60% de marcação; e 4, mais de 60% de células coradas. Para a intensidade de marcação foram dados valores de: 0, caracterizado por ausência de marcação; 1, marcação fraca; 2, marcação moderada; e 3, marcação forte. O valor final do escore variou de 0 a 12 e foi obtido pela multiplicação do valor da distribuição pelo da intensidade. Em seguida as amostras foram divididas em grupos de baixo (0-5) e alto (6-12) escores (LAVALLE *et al.*, 2012). A avaliação da expressão de VEGF se baseou na intensidade da marcação de imuno-histoquímica e a porcentagem de células tumorais positivas. Escore 0, caracterizado por completa ausência de marcação; 1, menos de 50% de células positivas com marcação fraca; 2, marcação fraca em mais de 50%

das células ou marcação forte em menos de 50% das células; 3, marcação forte em mais de 50% das células. Superexpressão de VEGF foi caracterizada como escores 2 e 3 e a não superexpressão de VEGF definido como escore 0 ou 1 (TSAI *et al.*, 2012). Reatividade para p63 foi indicada pela presença de distinta marcação nuclear e foi avaliada de forma semi-quantitativa usando o sistema de escore: (-) ausência de marcação; (+) marcação fraca ou <5% das células mioepiteliais positiva; (++) marcação moderada ou entre 5–50% das células mioepiteliais; (+++) marcação forte pelas células mioepiteliais (BERTAGNOLLI *et al.*, 2009).

Tabela 1.: Anticorpo, clone, fonte, diluição, recuperação antigênica, tempo e temperatura de incubação utilizados para a técnica de imuno-histoquímica.

Anticorpo (Clone)	**Fonte**	**Diluição**	**Recuperação Antigênica**	**Tempo (h) / Temperatura de Incubação**
Ki-67 (MIB-1)	Dakocytomation	1:25	Calor pressurizado (125°C/2min) com tampão citrato pH 6,0*	1/ Temperatura ambiente
ER (1D5)	Dakocytomation	1:20	Calor pressurizado (125°C/2min) com tampão EDTA pH 9,0*	1/ Temperatura ambiente
PR (HPRA2)	Neomarkers	1:20	Calor pressurizado (125°C/2min) com tampão EDTA pH 9,0*	1/ Temperatura ambiente
HER-2 (Polyclonal)	Dakocytomation	1:200	Banho maria (98°C/20min) com tampão citrato pH 6,0*	16/ 4°C
Cox-2 (SP21)	Neomarkers	1:80	Banho maria (98°C/20min) com tampão citrato pH 6,0*	1/ Temperatura ambiente
VEGF (Ab-1)	Neomarkers	1:200	Sem recuperação antigênica	1/ Temperature ambiente
p63 (4A 4)	Neomarkers	1:80	Banho maria (98°C/20min) com tampão citrato pH 6,0*	1/ Room temperature

***** (DakoCytomation Target Retrieval Solution)

6.8. Análise Estatística

6.8.1. Expressão de Marcadores Tumorais

A análise estatística foi realizada a partir do teste t de Student, teste Mann-Whitney e Correlação de Spearman. O coeficiente de correlação foi classificado como positivo ou negativo, e como fraco quando r, moderado quando $0.36 \leq r \leq 0.67$ e forte quando $r \geq 0.68$ (TAYLOR, 1990). Os valores serão considerados fortemente significativos quando $p < 0,01$, significativos quando $p < 0,05$ e tendendo para a significância quando $p < 0,10$.

6.8.2. Tempo de Sobrevida Global

O tempo de SG foi definido (em dias) como sendo o período entre a data da exérese cirúrgica do tumor até a data de óbito do animal que morreu pela doença. Os animais que vierem a óbito por razões desconhecidas ou causas não relacionadas ao tumor, ou que deixarem de ser acompanhados foram considerados censurados. A mediana de sobrevida dos animais foi definida como o período em que 50% dos pacientes de um determinado grupo vieram a óbito.

A SG dos animais foi avaliada em análise univariada, determinando o valor prognóstico do estadiamento clínico (TNM), tamanho tumoral, ulceração, tipo e grau histológicos, da expressão de Ki-67, RE, RP, Her-2, Cox-2, VEGF, assim como o valor terapêutico do tratamento adjuvante com carboplatina. As curvas de SG foram derivadas da estimativa de Kaplan-Meier. Pelo teste log-rank os valores foram considerados fortemente significativos quando $p < 0,01$, significativos quando $p < 0,05$ e tendendo para a significância quando $p < 0,10$.

7. RESULTADOS E DISCUSSÃO

Estes tópicos serão apresentados na forma de artigos científicos.

Artigo 1 - Submetido para o periódico *Journal of Veterinary Internal Medicine* (Anexo B) e formatado segundo as normas da revista.

Artigo 2 - Está sendo preparado para submissão.

Artigo 3 - Está sendo preparado para submissão.

ARTIGO 1

Journal of Veterinary Internal Medicine

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USE OF CARBOPLATIN IN FELINE MALIGNANT MAMMARY GLAND NEOPLASMS WITH ADVANCED CLINICAL STAGING

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USE OF CARBOPLATIN IN FELINE MALIGNANT MAMMARY GLAND NEOPLASMS WITH ADVANCED CLINICAL STAGING

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Short title: Carboplatin For Feline Mammary Carcinoma Treatment

Key words: Cat; Cancer; Chemotherapy; Surgery.

List of abbreviations:

UFMG: Federal University of Minas Gerais

FMCs: Feline Mammary Carcinomas

OS: Overall Survival

CETEA: Ethics Committee for Animal Experimentation

HE: Hematoxylin and Eosin

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This work was done at the Veterinary Hospital of the Veterinary School, Federal University of Minas Gerais (UFMG), and at the Laboratory of Comparative Pathology, Department of General Pathology, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), both in Belo Horizonte, Brazil.

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Abstract

Background: Feline mammary carcinomas (FMCs) are characterized by a guarded to poor prognosis and little progress has been made in extending patient survival.

Hypothesis/Objectives: The aim of this study was to compare overall survival (OS) periods of FMCs submitted to different treatment protocols, including surgery and adjuvant chemotherapy.

Animals: Sixteen queens admitted at the Veterinary Teaching Hospital of the Federal University of Minas Gerais, Brazil, were used in this study.

Methods: A retrospective analysis of conventional surgical excision alone or in association with adjuvant chemotherapy with carboplatin in patients classified as stage III and grade II or III was performed.

Results: Patients treated with surgery and chemotherapy presented a longer OS than those treated only with surgery, however, no statistical difference was observed when comparing both treatments (p=0.883).

Conclusions and clinical importance: Therapeutic benefit of carboplatin remains invalidated for FMCs and further investigation regarding adjuvant therapies are warranted.

Feline mammary neoplasms are the third most frequent tumor, following hematopoietic and cutaneous neoplasms.[1-4] The average age of diagnosis is 10 to 11 years,[2] and neutered animals are less likely to develop tumors than intact cats.[4] In contrast to humans and canines, at least 80% of all feline mammary tumors are malignant.[5,6] Furthermore, ulceration, lymphatic vessel invasion are common and regional or distant metastasis may be observed in over 80% of felines with malignant mammary neoplasms.[4]

Surgery remains the treatment of choice for feline mammary tumors[2] and may be used alone or in combination with chemotherapy or other modes of cancer therapy.[4] Chain mastectomy is the surgical method of choice, regardless of the size of the tumor, due to a reduction of local tumor recurrence, however with no increase in overall survival (OS) time.[1,4,7] One previous study found a significant difference in OS associated to surgical procedures, cats that received bilateral radical mastectomies had the longest survival times.[8] Early detection and aggressive treatment are notably important for feline mammary carcinomas (FMCs).[9] The response to chemotherapy may be poor once metastasis has occurred.[10]

Available chemotherapy protocols for feline mammary neoplasms consist of doxorubicin as a single agent or in combination with cyclophosphamide, carboplatin as a single agent or in combination with doxorubicin and the association of mitoxantrone with cyclophosphamide.[4,11,12] Additional clinical trials are needed to assess which chemotherapeutic doses and combinations are the most effective in increasing survival time.[10]

A previous study associated longer OS times for FMCs treated with surgery and adjuvant doxorubicin chemotherapy, although no control population was included.[8] Another study found similar median OS time, when treating FMCs with surgery, doxorubicin, and Cox-2 inhibitors; however it also lacked a control population.[13] No overall benefit to such adjuvant chemotherapy was observed in a different study that compared surgery plus chemotherapy to surgical treatment alone.[7] In addition, doxorubicin can be nephrotoxic in cats and careful evaluation of renal function is recommended.[4]

Little progress has been made in extending the survival time of patients with feline mammary tumors, characterized by a guarded to poor prognosis and median OS of less than one year.[3,4]

Therefore, the aim of this study was to compare OS periods of FMCs submitted to different treatment protocols, including surgery and adjuvant chemotherapy with carboplatin.

Material and methods

A retrospective evaluation of sixteen queens admitted at the Veterinary Teaching Hospital of the Federal University of Minas Gerais (UFMG), Brazil, was performed. The animals were divided into groups according to two different treatments protocols: 9 animals presenting FMCs submitted to surgical treatment alone through unilateral radical mastectomy; and 7 animals presenting FMCs submitted to conventional surgical excision and medication with four intravenous cycles of carboplatin[a] at a dose of 200mg/m^2, with a 21 day interval. Animals were treated solely with surgery mainly due to refusal of chemotherapy treatment.

The cases were staged according to a modified World Health Organization clinical staging system for feline mammary tumors. This system evaluates the tumor size (T_1: 0-2 cm; T_2: 2-3 cm; T_3: >3 cm), the neoplastic involvement of regional lymph nodes (N_0: no metastasis; N_1: metastasis) and the presence of distant metastasis (M_0: no metastasis; M_1: metastasis). Afterwards cases are divided into four stages: I ($T_1N_0M_0$), II ($T_2N_0M_0$), III ($T_3N_{0\text{-}1}M_0$, $T_{1\text{-}2}N_1M_0$), and IV ($T_{1,2,3}N_{0\text{-}1}M_1$).[7,13]

Tumor specimens were collected, fixed during 48 hours in 10% neutral buffered formalin solution and embedded in paraffin. Afterwards, 4 µm histological sections were obtained and stained with Hematoxylin and Eosin (HE). Tumors were classified according to veterinary histological criteria.[6]

Histological grade of the tumors was established according to the Nottingham system.[14] This system evaluates tubule formation index (1 point: more than 75% of the tumor is composed

by tubules, two points: between 10% and 75% of tubular formations, and 3 points: the tubules occupy 10% or less of the tumor), nuclear pleomorphism (1 point: small and regular nuclei; 2 points: moderate increase in size and variation of nuclei; 3 points: marked pleomorphism, with large variation in size and shape of nuclei) and mitotic count (1 point: 0-8 mitosis, 2 points: 9-16 mitosis, and 3 points: above 17 mitosis in 40x lens). The assignment of points was carried out using an Olympus BX-40 microscope fitted with a 10x eyepiece and a 40x objective. This gives a field area of 0.239 mm^2. Histological grade of the tumor is obtained through the sum of the scores which results in a total amount that ranges from 3 to 9. Afterwards, the tumor is classified as grade I (3-5 points), grade II (6-7 points), and grade III (8-9 points).

Patient follow-up was obtained through the evaluation of patient medical records and telephone interviews to owners in order to evaluate disease evolution with possible recurrences and metastases. In order to select more uniform cases, queens were excluded from the analysis when tumors were initially diagnosed as grade I, or classified as stage I, II, or IV.

OS time was defined as the period (in days) between the date of surgical removal of the tumor and death caused by the disease. Animals that died from unknown causes or causes unrelated to the tumor were censured. OS was evaluated by univariated analysis (Kaplan-Meier estimated survival curves). Values were considered statistically significant when $P<0.05$ by the Log-rank Test (Cox-Mantel)[b]. Median survival was defined as the period when 50% of patients of a determined group died.

All procedures were performed under the appropriate guidelines and with the approval of the Ethics Committee for Animal Experimentation (CETEA/UFMG), protocol 13412/2012.

Results

The 16 FMCs evaluated were histologically diagnosed as: seven (43.75%) cribriform carcinomas, four (25.00%) tubulopapillary carcinomas, one (6.25%) papillary carcinoma, one (6.25%) micropapillary carcinoma, one (6.25%) solid carcinoma, one (6.25%) tubular carcinoma, and one (6.25%) glycogen rich carcinoma.

Regarding histological grade, 16 invasive carcinomas were evaluated as: ten (62.50%) grade II, and six (37.50%) grade III. All (100.00%) patients evaluated for clinical staging were classified as stage III.

Carboplatin administration was well tolerated by patients. Minimal side-effects were observed, such as myelossupression at the drug`s nadir period and occasional and discreet gastrointestinal complications.

Median patient follow-up time was 202 days (ranging from 1 to 1400 days). Among patients submitted only to the surgical treatment, four (44.45%) died due to the progression of the FMC, three (33.33%) died due to other reasons, and two (22.22%) remained alive. Among patients submitted to conventional surgical excision and adjuvant chemotherapy with carboplatin, five (71.44%) died due to the progression of the FMC, one (14.28%) died due to other reasons, and one (14.28%) remained alive.

Patients treated only with surgery presented a median survival of 387 days while those treated with surgery and chemotherapy presented a median survival of 428 days. No statistical difference was observed when comparing the OS of the two different treatment groups, with $p=0.883$ (Fig 1).

Discussion

The present study evaluated OS associated to surgical treatment alone or with the addition of carboplatin in a more homogeneous sample. Chemotherapy did not present clinically important side-effects, however, no significant statistical difference was observed in the OS of both treatments. The guarded to poor prognosis and short OS of FMCs generates a necessity for studies involving new adjuvant treatments that may enable longer OS. Carboplatin has been indicated for such treatment, however clear benefit remains to be found.

Tubular, papillary, solid, and cribriform carcinomas are described as the most frequent invasive histological types found in the feline mammary gland, and some carcinomas show a combination of histologic types in one lesion.[4,10] Although the studied patients presented diversified malignant histological types, a poor prognosis was associated to all cases due to moderate or poor differentiation and advanced clinical staging. To the authors' knowledge, this is the first study that attempts to include a more uniform population in control and treated with adjuvant chemotherapy groups. In addition, previous studies failed to maintain a standard protocol for chemotherapy (including dosage, association of other drugs, and number of cycles),[7,8,13] and in the present study queens included in each group received identical treatments.

The Elston and Ellis method is the most common method for histological grading of invasive carcinomas, and is strongly correlated to prognosis.[15] FMCs are mainly classified as moderately or poorly differentiated,[10,15,16] as was found in this study.

In this study, most cases (93.75%) were clinically staged as stage III, characterized by tumors larger than 3 cm and/or regional lymph node metastasis. Feline mammary tumors larger than 3 cm are associated with a poor prognosis by several authors.[1,4,9,12,13,17] Lymphovascular

invasion and lymph node metastases were significantly associated with survival and considered independent prognostic predictors.[15]

Carboplatin is indicated for the treatment of canine and feline malignant mammary gland neoplasms.[11] Adjuvant chemotherapy with carboplatin has been proven to be beneficial in the treatment of dogs with canine mammary tumors with advanced clinical staging.[18] The drug was also used as a rescue therapy agent alone or in association to other chemotherapy drugs (mitoxantrone and doxorubicin) for FMCs presenting recurrences or metastases.[8] In this study, although the patients treated with adjuvant chemotherapy presented longer median OS, no significant statistical difference was observed when comparing the OS of feline patients treated with only with surgery or surgery associated to carboplatin. This could be due to some limitations of the design of the study, characterized by a retrospective and nonrandomized analysis with a small number of animals. The OS of both treatment groups were higher than described in the literature,[3,4] demonstrating the efficacy of the chosen surgical treatment.

Conclusion

Unilateral radical mastectomy promotes an important increase in overall survival. However, therapeutic benefit of carboplatin remains invalidated for FMCs. Randomized, prospective clinical trials with a larger number of patients are warranted for further investigation regarding adjuvant therapies for feline mammary neoplasms that may enable longer OS.

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Endnotes

[a] Carboplatin; B-platin®; active ingredient: 150mg; excipient: water for injection; Blausiegel Ind. e Com. Ltda; Avenue Ivo Mário Isaac Pires, 7602, Cotia, São Paulo, Brazil .

[b] GraphPad Prisms v. 5.0; GraphPad, San Diego, CA, USA

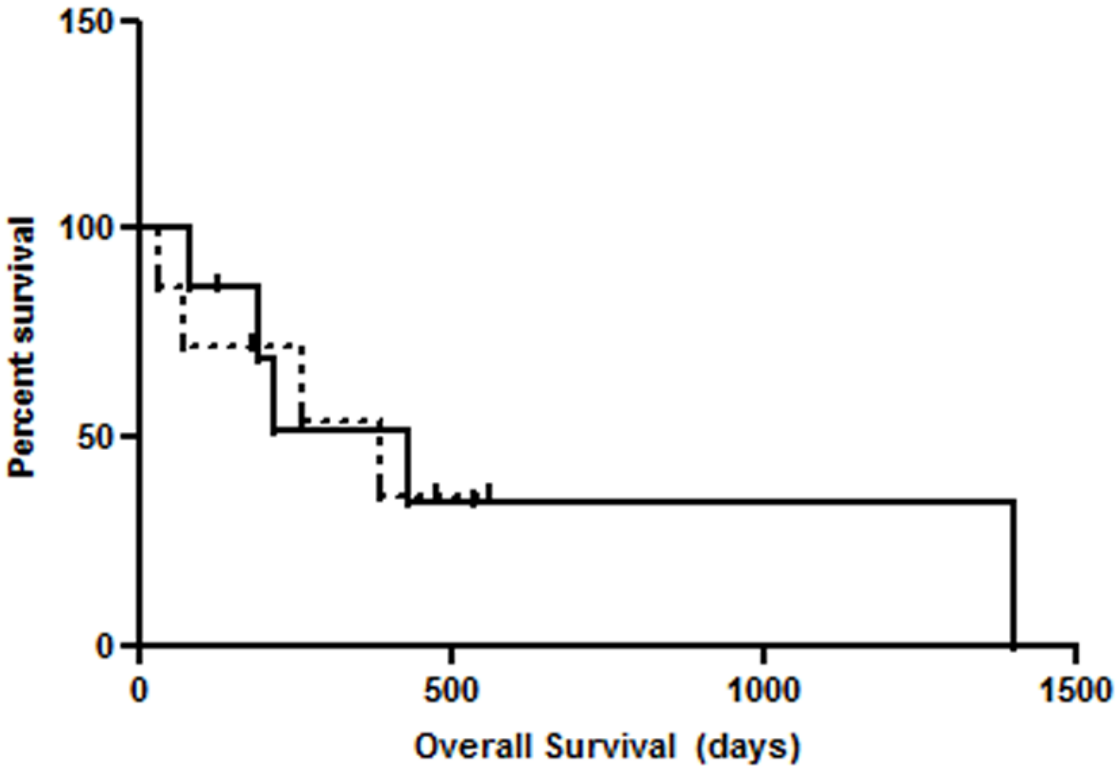

Figure 1. Overall survival curves for 16 female cats according to therapy. Patients submitted to surgical treatment alone, 9 cases (-----); and conventional surgical excision and carboplatin, 7 cases (──) (p=0.883).

ARTIGO 2

EVALUATION OF PROGNOSTIC FACTORS AND SURVIVAL RATES IN MALIGNANT FELINE MAMMARY GLAND NEOPLASMS

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Abstract

Mammary neoplasms are the third most frequent tumor in felines and at least 80% are malignant. Several prognostic factors have been described for feline mammary gland neoplasms and their understanding may enable individualized therapeutic strategies and possible increase in overall survival (OS). The aim of this paper is thus to investigate the association of prognostic factors suggested in the literature for feline mammary gland neoplasms, correlating them with OS. The primary malignant feline mammary gland neoplasms (MFMGN) and lymph nodes of 37 queens were evaluated in a retrospective manner. Clinical staging, histological type and grade, OS and immunohistochemical analysis for Ki-67, ER, PR, Her-2, Cox-2, and VEGF were obtained. Lymph node metastasis was found in 35% of cases, the majority was classified as stage III, tubulopapillary carcinomas, and grade II. Neoplasms positive for hormonal receptors, negative for Her-2, and with VEGF overexpression prevailed. Immunoreactivity for Ki-67 (p=0.046) and Cox-2 (p=0.007) was higher in metastases when compared to primary tumors. Cox-2 (p=0.089), Her-2 (p=0.012), and histological grade (p=0.080) were correlated to OS. Data suggest that inhibition of ovarian hormones and Cox-2 may represent a therapeutic option for MFMGN. Cox-2 scores and Ki-67 index should be analyzed in primary tumors and metastases, and histological grade, Her-2 status, and Cox-2 scores had direct influence in OS.

Introduction

Following hematopoietic and cutaneous neoplasms, feline mammary neoplasms are the third most frequent tumor (MACEWEN *et al*., 1984; MISDORP, 2002; OVERLEY *et al*., 2005; LANA *et al*., 2007). Neutered animals are less likely to develop tumors than intact cats (LANA *et al*., 2007). In contrast to humans and canines, at least 80% of all feline mammary tumors are malignant (BOSTOCK, 1986; MISDORP *et al*., 1999). Ulceration, lymphatic vessel invasion are common and regional or distant metastasis may be observed in over 80% of malignant feline mammary gland neoplasms (LANA *et al*., 2007).

Prognostic factors are clinical, pathologic, and biologic features of cancer patients and their tumors that forecast clinical outcome (ALLRED *et al*., 1998). The most significant prognostic factors for malignant feline mammary gland neoplasms (MFMGN) are the tumor size, extent of surgery, and histological grade (LANA *et al*., 2007). Other factors that influence in

disease-free interval and overall survival (OS) are: clinical staging, histological subtype, mitotic index, development of metastatic disease, location of metastatic disease. Molecular markers have also been studied for feline mammary neoplasms, including: human epidermal growth factor receptor type 2 (Her-2), vascular endothelial growth factor (VEGF), Cyclooxygenase enzymes (COX), Ki-67, and estrogen and progesterone receptors (ER and PR) (GIMENÉZ *et al.*, 2010; NOVOSAD *et al.*, 2006; HUGHES; DOBSON, 2012).

The understanding of prognostic factors associated to the biological behavior of neoplastic diseases enables individualized therapeutic strategies aiming towards an increase in disease free interval and OS. The aim of this paper is thus to investigate the association of prognostic factors suggested in the literature for malignant feline mammary gland neoplasms, correlating them with overall survival.

Material and Methods

Thirty-seven female cats admitted at the Veterinary Teaching Hospital of the Federal University of Minas Gerais, Brazil, and diagnosed with MFMGN were evaluated in a retrospective manner. Fifty-six MFMGN and sixteen metastatic regional lymph nodes samples were analyzed. Samples were obtained from the Pathology Sector of the Clinical and Surgical Department of the Veterinary School and the Laboratory of Comparative Pathology in the Institute of Biological Sciences, both at the Federal University of Minas Gerais, Brazil.

The cases were staged according to a modified World Health Organization (WHO) clinical staging system for feline mammary tumors. This system evaluates the tumor size (T_1: 0-2 cm; T_2: 2-3 cm; T_3: >3 cm), the neoplastic involvement of regional lymph nodes (N_0: no metastasis; N_1: metastasis) and the presence of distant metastasis (M_0: no metastasis; M_1: metastasis). Afterwards cases are divided into four stages: I ($T_1N_0M_0$), II ($T_2N_0M_0$), III ($T_3N_{0-1}M_0$, $T_{1-2}N_1M_0$), and IV ($T_{1,2,3}N_{0-1}M_1$) (MCNEILL *et al.*, 2009; BORREGO *et al.*, 2009).

Tumour specimens were collected, fixed during 48 hours in 10% neutral buffered formalin solution and embedded in paraffin. Histological sections of 4 µm were obtained and stained with Hematoxylin and Eosin (HE). Tumors were reviewed and reclassified according to veterinary histological criteria (MISDORP *et al.*, 1999) and tumors displaying multiple morphological patterns were classified according to the predominant neoplastic pattern.

Histological grade of the invasive carcinomas was established according to the Nottingham system (ELSTON; ELLIS, 1998). This system evaluates tubule formation index (1 point: more than 75% of the tumor is composed by tubules, two points: between 10% and 75% of tubular formations, and 3 points: the tubules occupy 10% or less of the tumor), nuclear pleomorphism (1 point: small and regular nuclei; 2 points: moderate increase in size and variation of nuclei; 3 points: marked pleomorphism, with large variation in size and shape of nuclei) and mitotic count (1 point: 0-8 mitosis, 2 points: 9-16 mitosis, and 3 points: above 17 mitosis in 40x lens). The assignment of points was carried out using an Olympus BX-40 microscope fitted with a 10x eyepiece and a 40x objective. This gives a field area of 0.239 mm^2. Histological grade of the tumor was obtained through the sum of the scores which results in a total amount that ranges from 3 to 9. Afterwards, the tumor was classified as grade I (3-5 points), grade II (6-7 points), and grade III (8-9 points).

Following case revision, 4 μm histological sections were obtained for immunohistochemical analysis. Details related to target antigen, clone, manufacturer, dilution, antigen retrieval method and incubation time are described in Table 1. Immunohistochemical procedures were identified using secondary antibodies (Advance HRP, DakoCytomation). Endogenous peroxidase activity was blocked with a solution of 3% H_2O_2 in methyl alcohol. Reagents were applied for 30 minutes manually and immunoreactivity was visualised by incubating the slides for 10 minutes with diaminobenzidine (DAB Substrate System; Dakocytomation). Sections from a known Ki-67, Her-2, Estrogen Receptor (ER), and Progesterone Receptor (PR) positive feline mammary carcinoma, sections from a human colon carcinoma known to express COX-2, and sections from a human mammary gland neoplasm carcinoma known to express VEGF were used as positive controls. Negative controls were assessed using normal serum (Ultra V Block, Laboratory Vision) as the primary antibody.

The proliferative index was calculated by counting the number of positive nuclei for Ki-67 staining in a total of 1000 neoplastic cells from each lesion (400x magnification) (DUTRA *et al.*, 2004). Staining for ER and PR was evaluated, and cases were scored positive if nuclear staining was present in 1% or more of the tumour cells (HAMMOND *et al.*, 2010). Her-2 expression was determined by epithelial cell membrane staining and scored according to the guidelines established by the American Society of Clinical Oncology, College of American Pathologists (ASCO/CAP). Samples were classified into four categories: negative with score

0 or 1+, indeterminate with score 2+, and positive with score 3+. Score 0 is characterized by absent membrane staining; 1+ weak, incomplete membrane staining of any proportion of the tumor cells; 2+ complete membrane staining that is either non-uniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells, or intense, complete membrane staining of 30% or fewer tumor cells; 3+ complete, uniform, intense membrane staining of > 30% of tumor cells. Her-2 overexpression was only considered in samples presenting 3+ scores (WOLFF *et al*., 2007). Positivity for COX-2 was indicated by the presence of cytoplasmatic staining. The number of positive COX-2 cells was evaluated semi quantitatively with the distribution score defined by the estimated percentage of positive cells in five microscope fields (magnification 400x): 0 = absence, 1 = fewer than 10% of stained cells, 2 = between 10% and 30%, 3 = 31% and 60%, 4 = more than 61% of stained cells. For staining intensity, values from 0 to 3 were attributed: 0 = absence, 1 = weak marking, 2 = moderate marking, and 3 = strong marking. Distribution scores and intensity were multiplied to obtain the total score, which ranges from 0 to 12, and then divided into groups of low (0-5) and high (6-12) scores (LAVALLE *et al*., 2012). The scoring of VEGF expression was based on the intensity of IHC staining and the percentage of positive cancerous cells. A score 0 represents a complete lack of staining, score 1 represents less than 50% positive cells with weak staining, score 2 represents weak positive staining in more than 50% of cells or strong staining in less than 50% of cells, score 3 represents strong positive staining in more than 50% of cells. VEGF overexpression was defined as score 2 and 3, while VEGF non-overexpression was defined as score 0 or 1 (TSAI *et al.*, 2012).

Patient follow-up was obtained through the evaluation of medical records and telephone interviews to owners in order to evaluate disease evolution with possible recurrences, metastases, and death. Due to the retrospective nature of this study, some medical records were incomplete, lacking clinical information. Furthermore, some immunohistochemical techniques could not be analyzed in specific cases, probably mainly due to differences in antigen preservation.

Table 1.: Target antigen, clone, manufacturer, dilution, antigen retrieval method and incubation time and temperature for immunohistochemical analysis for Ki-67, Estrogen Receptor (ER), Progesterone Receptor (PR), Cyclooxigenase-2 (Cox-2), Vascular Endothelial Growth Factor (VEGF), and Human Epidermal Growth Factor Receptor type 2 (Her-2) in malignant feline mammary gland neoplasms.

Target Antigen (Clone)	Manufacturer	Dilution	Antigen Retrieval Method	Incubation Time (h) / Temperature
Ki-67 (MIB-1)	Dakocytomation	1:25	Pressurised Heat (125°C/2min) with citrate buffer pH 6.0*	1 / Room Temperature
RE (1D5)	Dakocytomation	1:20	Pressurised Heat (125°C/2min) with EDTA buffer pH 9.0*	1 / Room Temperature
RP (HPRA2)	Neomarkers	1:20	Pressurised Heat (125°C/2min) with EDTA buffer pH 9.0*	1 / Room Temperature
Her-2 (Polyclonal)	Dakocytomation	1:200	Water bath (98°C/20min) with citrate buffer pH 6.0*	16 / 4°C
Cox-2 (SP21)	Neomarkers	1:80	Water bath (98°C/20min) with citrate buffer pH 6.0*	1 / Room Temperature
VEGF (Ab-1)	Neomarkers	1:200	No antigen retrieval	1 / Room Temperature

*(DakoCytomation Target Retrieval Solution)

OS time was defined as the period (in days) between the date of surgical removal of the tumor and death caused by the disease. Animals that died from unknown causes or causes unrelated to the tumor were censored. Median survival was defined as the period when 50% of the patients of a determined group had died.

Statistical analyses were performed with Student's t-test, Mann-Whitney U test, and Spearman's Rank Correlation Coefficient. The correlation coefficient was considered positive or negative, and weak when $r \leq 0.35$, moderate when $0.36 \leq r \leq 0.67$, and strong when $r \geq 0.68$ (TAYLOR, 1990). Overall survival time was evaluated by univariate Kaplan-Meier estimated survival curve analysis by the log-rank test (Cox-Mantel). Results for all testing procedures were considered significant highly significant when $p \leq 0.01$, significant when $p \leq 0.05$, and trending towards significance when $p \leq 0.10$.

All procedures were performed under the appropriate guidelines and with the approval of the Ethics Committee for Animal Experimentation (CETEA/UFMG), protocol 13412/2012.

Results

Thirty-seven feline patients were analyzed. Thirteen (35.13%) animals presented more than one primary neoplasm, resulting in 56 MFMGN. At the time of initial diagnosis, ten (27.02%) animals presented one, and three (8.11%) animals presented two metastatic regional lymph nodes (axillary and / or inguinal), resulting in 16 samples of metastases (Fig. 1A). The primary neoplasms were classified as: six (10.72%) carcinomas *in situ* and 50 (89.28%) invasive malignant neoplasms.

Neoplasms were found mostly in abdominal mammary glands (28/39; 71.80%) then in thoracic mammary glands (11/39; 28.20%). Skin ulceration associated to tumors was found in 18.60% of studied cases. Neoplasm size was classified as less than 2 cm in 20/44 (45.45%) cases, 2-3 cm in 11/44 (25.00%) cases, and over 3 cm in 13/44 (29.55%) cases. Evaluation of clinical staging demonstrated 17/25 (68.00%) stage III, 4/25 (16.00%) stage II, and 4/25 (16.00%) stage I tumors.

The 56 primary MFMGN were diagnosed as: 19 (33.93%) tubulopapillary carcinomas (Fig. 1B), 14 (25,00%) cribriform carcinomas (Fig. 1C), six (10.71%) *in situ* carcinomas, four (7.14%) papillary carcinomas, three (5.36%) mucinous carcinomas, two (3.58%) solid carcinomas, two (3.58%) tubular carcinomas, two (3.58%) glycogen rich carcinomas, one (1.78%) micropapillary carcinoma, one (1.78%) carcinosarcoma, one (1.78%) malignant adenomyoepithelioma, and one (1.78%) carcinoma in mixed tumor. Regarding histological grade, 49 invasive carcinomas were analyzed and considered as: 13 (26.53%) grade I, 24 (48.98%) grade II, and 12 (24.49%) grade III.

Immunohistochemistry for Ki-67 antibody demonstrated a mean labeling of 24.15 ± 18.28% (range: 2.00%; 65.40%) in primary tumors and 34.22 ± 16.42% (range: 2.00%; 62.80%) in regional metastases (Fig. 1D). Estrogen receptor immunolabeling was positive in 38/43 (88.37%) and negative in 5/43 (11.63%) MFMGN. ER status was positive in 11/14 (78.57%) and negative in 3/14 (21.43%) lymph nodes (Fig. 1E). All 55/55 (100.00%) primary neoplasms and 16/16 (100.00%) regional metastases analyzed cases were positive for

progesterone receptor (Fig. 1F). Evaluation of immunohistochemistry for Her-2 showed 33/51 (64.71%) score 1+ (Fig. 2A), 13/51 (25.49%) score 2+, 5/51 (9.80%) score 3+ (Fig. 2B) in primary tumors and 10/16 (62.50%) score 1+, 4/16 (25.00%) score 2+, 2/16 (12.50%) score 3+ in regional metastases. Cox-2 immunolabeling in 55 MFMGN cases demonstrated 7/55 (12.72%) score 1, 12/55 (21.82%) score 2, 9/55 (16.36%) score 3, 11/55 (20%) score 4, 10/55 (18.18%) score 6, 4/55 (7.28%) score 8, 2/55 (3.64%) score 9, and 0/55 (0.00%) score 12. These cases were then divided into 39/55 (70.91%) low (Fig. 2C) and 16/55 (29.09%) high scores (Fig. 2D). The evaluated metastases presented 0/16 (0.00%) score 1, 3/16 (18.75%) score 2, 1/16 (6.25%) score 3, 1/16 (6.25%) score 4, 5/16 (31.25%) score 6, 4/16 (25.00%) score 8, 1/16 (6.25%) score 9, and 1/16 (6.25%) score 12. Cases were divided into 5/16 (31.25%) low and 11/16 (68.75%) high Cox-2 scores. VEGF immunolabeling demonstrated 13/52 (25.00%) score 1 (Fig. 2E), 25/52 (48.08%) score 2, and 14/52 (26.92%) score 3 (Fig. 2F) in evaluated MFMGN, afterwards divided into 39/52 (75.00%) presenting VEGF superexpression and 13/52 (25.00%) without superexpression. VEGF expression in regional metastases demonstrated 4/16 (25.00%) score 1, 9/16 (56.25%) score 2, and 3/16 (18.75%) score 3, afterwards divided into 12/16 (75.00%) presenting VEGF superexpression and 4/16 (25.00%) without superexpression.

When comparing primary tumors and metastases, statistically significant differences were observed in Cox-2 and Ki-67 immunostaining. Median Cox-2 score was 3 (range: 1;9) in primary tumors and 6 (range: 2;12) in lymph node metastases (p=0.007). Mean Ki-67 expression in primary tumors was 24.15% and 34.22% in regional metastases (p=0.046).

Immunohistochemical staining for Ki-67 was correlated with histological grading (r=0.260; p=0.081), tumor size (r=0.341; p=0.022), and the progressive types of diagnosis (*in situ* carcinomas, invasive carcinomas and metastases) (r=0.322; p=0.007). Her-2 was correlated with tumor size (r=-0.355;p=0.020). Correlations were also found among Cox-2 and VEGF (r=0.204; p=0.094), and Cox-2 and progressive types of diagnosis (*in situ* carcinomas, invasive carcinomas and metastases) (r=0.359; p=0.002). Ki-67 expression in tumors with low Cox-2 scores was lower (23.07%) than in tumors with high Cox-2 scores (32.09%) (p=0.041). TNM clinical staging was correlated to tumor size (r=0.339; p=0.026).

Statistically significant differences were observed in ER negative tumors expressing higher Her-2 scores (median 2, range: 1;3) than ER positive tumors (median 1, range: 1;3) (p=0.041). A significant difference was observed regarding the presence of ulceration and larger tumors (median T_3, range: T_2;T_3) while smaller tumors (median T_1, range: T_1;T_3) did not present skin discontinuity (p=0.001). Tumors presenting overexpression of VEGF presented higher Cox-2 scores (median 4, range:1;12) than tumors non-overexpressing VEGF (median 3, range:1;8) (p=0.019).

A statistically significant difference in overall survival was observed when comparing high and low Cox-2 scores. Patients with high Cox-2 scores presented a median survival of 189 days while low Cox-2 scores presented a median survival of 1400 days (p=0.089) (Fig. 3). Regarding Her-2 expression, the median survival of patients presenting score 1+ was 262 days, 387 days for score 2+ and 35 days for score 3+ (p=0.012) (Fig. 4). Histological grade also had an impact on overall survival. Grade I tumors had an median survival of 1628 days, 262 days for grade II tumors, and 78 days for grade III tumors (p=0.080) (Fig. 5).

Discussion

Multiple tumors of the same or different histological types are generally observed in 40% of feline patients presenting mammary neoplasms, similar to findings of this study. The incidence of benign tumors in cats is 20%, while 80% are malignant (MISDORP *et al*., 1999; MISDORP, 2002), demonstrating the aggressive character of feline mammary neoplasms. An increased rate of lymph node metastasis was observed in the present study (35.13%) when compared to the 27% described by Misdorp *et al*. (1999), this result was similar to the 38.5% of regional metastasis described by Novosad *et al*. (2006).

Most tumors (64%) in the present study were classified as stage III, and 16% were classified as stage I or II. Novosad *et al*. (2006) and Borrego *et al*. (2009) demonstrated a more homogeneous distribution (31% stage I, 20% stage II, and 49% stage III; and 30% grade I, 28% grade II, and 42% grade III, respectively), although stage III prevailed. An approximate ratio of 7:3 was observed in the incidence of tumors in the abdominal and thoracic mammary glands, respectively, in the present study and by Borrego *et al*. (2009). Novosad *et al*. (2006) described 14.2%, and Borrego *et al*. (2009) described 21% of ulcerated tumors at the time of evaluation, similar to the 18.6% found in this study. Novosad *et al*. (2006) also found an

association between ulceration and clinical stage, which was not found in this study. Novosad *et al*. (2006) described tumor sizes very similar to the present study, 40% of tumors <2 cm in diameter, 27% had tumors 2 to 3 cm in diameter, and 23% had tumors >3 cm in diameter. Borrego *et al*. (2009) demonstrated a smaller proportion of tumors larger than 3 cm (13%), which may possibly be related to shorter clinical evolution of the disease. The correlation between TNM staging and tumor size is expected since the latter is evaluated in clinical staging. Rapid growth often is accompanied by superficial ulceration (MODIANO *et al*., 2007), which justifies the correlation found between tumor size and ulceration.

Histological types found in this study were diversified. Tubular, papillary, solid, cribriform, and *in situ* carcinomas are considered as common diagnosis in the feline mammary gland, and some carcinomas show a combination of histologic types (MISDORP *et al*., 1999; MISDORP, 2002; LANA *et al*., 2007), similar to described in this study. Some histologic types found in this study were described as rare in the feline mammary gland, such as the mucinous carcinoma, carcinoma in mixed tumor, and carcinosarcoma (MISDORP *et al*., 1999; MISDORP, 2002).

The Elston and Ellis method was described as a reliable and independent prognostic factor for MFMGN evaluating the biological aggressiveness of the disease with high prognostic value (SEIXAS *et al*., 2011). MFMGN are mainly classified as moderately differentiated (CASTAGNARO *et al*., 1998; MILLANTA *et al*., 2002a; MILLANTA *et al*., 2002b; DIAS PEREIRA *et al*., 2004; MILLANTA *et al*., 2005a; MILLANTA *et al*., 2005b; MILLANTA *et al*., 2006; SEIXAS *et al*., 2011), as was found in this study. The prognostic value of histological grade in MFMGN was demonstrated when shorter overall survival is associated to higher histological grade (SEIXAS *et al*., 2011; MILLANTA *et al*., 2002a), as was found in this study. Our study correlated higher Ki-67 index with higher histological grade, which is in accordance with previous reports (SEIXAS *et al*., 2011; DIAS PEREIRA *et al*., 2004). Millanta *et al*. (2002a) did not show such correlation, which is unexpected since histological grading includes mitosis count and loss of differentiation is generally associated to higher growth rates.

Ki-67 labeling index is an excellent marker for determining the growth fraction of a given population (MILLANTA *et al*., 2002a). Rasotto *et al*. (2011) found similar proliferative index

(29.8%) as the present study (24.15%). Our data demonstrates a statistical significant difference of proliferation index in primary tumors and regional metastases. Furthermore, Ki-67 immunolabeling was positively correlated to progressive types of diagnosis (including *in situ* carcinomas, invasive carcinomas and lymph node metastases). Millanta *et al*. (2002a) demonstrated higher Ki-67 index in invasive carcinomas when compared to *in situ* carcinomas. Dias Pereira *et al.* (2004) described lower Ki-67 index in metastasis when compared to the primary tumor, although only two cases were evaluated. Previous studies (MILLANTA *et al*., 2002a; DIAS PEREIRA *et al*. 2004; RASOTTO *et al*., 2011) report a progressive increase in proliferative index from normal mammary gland, non-neoplasic lesions, benign neoplasms, *in situ* carcinomas, and invasive carcinomas. Although significant samples of lymph node metastases were not included in these studies, our results demonstrate that metastatic lesions represent a further step in tumor progression, with higher Ki-67 proliferation index. Our correlation between proliferation index and histological grade was also observed by Dias Pereira *et al.* (2004), contrasting earlier work (MILLANTA *et al*., 2002a). In addition, we also correlated Ki-67 expression to tumor size, indicating more aggressive biological behavior in larger tumors.

Results for estrogen and progesterone receptor in the present study diverged from literature findings (LAS MULAS *et al*., 2000; LAS MULAS *et al*., 2002; MILLANTA *et al*., 2005a; LANA *et al*., 2007), contradicting the proposal of feline mammary neoplasms as models for hormone-independent human breast cancer. All primary neoplasms and metastases were positive for PR and 88.37% of primary neoplasms and 78.57% of lymph node metastases were positive for ER. This finding could be due to different methodologies applied. In our study the ASCO/CAP guideline recommendations for immunohistochemical testing of ER and PR in breast cancer was used, and tumors were considered positive when at least 1% of positive tumor nuclei were observed, which may increase the number of positive cases, when compared to previous studies that negative cut-off was established at 5% (LAS MULAS *et al*., 2000; LAS MULAS *et al*., 2002; MILLANTA *et al*., 2005a). However, Millanta *et al*. (2006) found 91.4% of positivity for PR. The role of ovarian hormones in feline mammary neoplasms is evidenced by loss of protection from the disease with increasing hormonal exposure, overrepresentation of intact cats diagnosed with mammary tumors (OVERLEY *et al*., 2005), and sporadic occurrence of mammary lesions concomitant with synthetic progestins (HAYDEN *et al*., 1989). This finding may be relevant to suggest endocrine

therapies (ET) for MFMGN. In women with breast cancer characterized as endocrine responsiveness, ET seems probable to be effective in improving disease-free and overall survival. Some treatment options include: ovarian ablation, aromatase inhibitors, and tamoxifen (GOLDHIRSCH *et al.*, 2005).

In human oncology, many studies have demonstrated that *HER-2* amplification and / or overexpression of its receptor are linked to shorter overall disease-free intervals, increased risk of metastasis, and resistance to many types of therapy (MILLANTA *et al.*, 2005b). In our study, contradicting previous findings (MILLANTA *et al.*, 2005b; RASOTTO *et al.*, 2011), Her-2 immunolabeling was mainly classified as score 1+. Some lack of standardization of methodologies employed may justify the discrepancy between approximately 10% of Her-2 overexpression in primary tumors in our study and 57% by Millanta *et al*. (2005b), that considered scores 2+ and 3+ as overexpression of Her-2. Rasotto *et al.* (2011) reported only 5.5% of Her-2 overexpression, also considered as scores 2+ and 3+. Controversies regarding the incidence of Her-2 expression in MFMGN could also be the result of subjective interpretation of Her-2 immunolabeling (ORDÁS *et al.*, 2007; RASOTTO *et al.*, 2011). As described in human breast cancer (QUÉNEL *et al.*, 1995) and feline mammary lesions (ORDÁS *et al.*, 2007), higher Her-2 scores were more frequently expressed in ER negative tumors in the present study, both characteristic of poor prognosis. In addition, contradicting human breast literature (SWEDE *et al.*, 2003; AZIZUN-NISA *et al.*, 2008) and previous findings in feline mammary lesions (ORDÁS *et al.*, 2007), Her-2 expression scores were higher in smaller tumors. The prognostic importance of Her-2 expression is demonstrated with different overall survival associated to immunohistochemical status (MILLANTA *et al.*, 2005b), as seen in the present study.

Cyclooxigenase-2 (Cox-2) is a rapidly inducible enzyme, involved in the process of malignant transformation and tumor progression by affecting cell proliferation, mitosis, cell adhesion, apoptosis, immune-surveillance, and angiogenesis (WILLIAMS *et al.*, 1999; MILLANTA *et al.*, 2006). Previous studies reported high percentages (87% and 96%) of MFMGN expressing Cox-2 (SAYASITH *et al.*, 2009; MILLANTA *et al.*, 2006), in our study, all MFMGN expressed variable Cox-2 scores. This indicates that Cox-2 presents some role in feline mammary carcinogenesis (SAYASITH *et al.*, 2009). To the author's knowledge, this is the first study evaluating Cox-2 expression in feline primary tumors and metastases. Statistically

significant differences were observed in Cox-2 scores when comparing primary tumors and regional metastases, and *in situ* carcinomas, invasive carcinomas, and lymph node metastases. Therefore, the present study reinforces the previous statement by Sayasith *et al*. (2009) that Cox-2 is a potential target for therapeutic and preventive strategies and support efforts to evaluate clinical trials on the efficacy of Cox-2 inhibitors in the treatment of MFMGN. Borrego *et al*. (2009) did not find any clear prognostic advantage in treating MFMGN with Cox-2 inhibitor in addition to surgery and chemotherapy. However, clinical cases presenting metastasis, and consequentially higher Cox-2 scores, could potentially benefit more from such treatment. In addition, clinical trials should select patients presenting high immunohistochemical Cox-2 scores for treatment with Cox-2 inhibitors. Our results differ from a previous study (MILLANTA *et al*., 2006), associating higher Cox-2 scores with high Ki-67 proliferation index, justified by cell proliferation stimulation by Cox-2. Cox-2 immunolabeling was also associated to VEGF imunolabeling, a potent angiogenic growth factor. Millanta *et al*., (2006) justify this relationship due to a possible promotion and support of angiogenesis by Cox-2. Elevated Cox-2 expression associated to poor prognosis is clearly demonstrated through differences in overall survival, described by our study and Millanta *et al*. (2006).

Vascular endothelial growth factor (VEGF) is a potent anigiogenic factor envolved in tumor growth, invasion, and metastasis (MILLANTA *et al*., 2002b; GIMENEZ *et al*., 2010; TSAI *et al*., 2012). In feline mammary tumors, Millanta *et al*. (2002b) found VEGF expression in all investigated carcinomas and related to prognosis and clinical outcome. Our results demonstrated predominant score 2 immunostaining, in primary tumors and regional metastases, and most tumors were associated to VEGF superexpression. However, no difference in overall survival or prognostic value was associated to VEGF. This might be due to different methodologies employed for VEGF expression scoring.

Conclusion

The understanding of malignant feline mammary gland neoplasms, as well as increases in overall survival rates, is warranted. Aggressive surgical excision remains the main treatment option, and additional therapeutic interventions are necessary. The study of prognostic factors revealed that some of these factors may be predictive seen that treatments involving the

inhibition of ovarian hormones and cyclooxigenase-2 enzyme may possibly represent a therapeutic option for MFMGN. When evaluating disease progression, Cox-2 scores and Ki-67 index should be analyzed in primary tumors and metastases. Histological grade, Her-2 overexpression, and Cox-2 scores were found to influence directly in overall survival of queens. Studies involving MFMGN should employ similar and strict methodologies, to enable data comparison.

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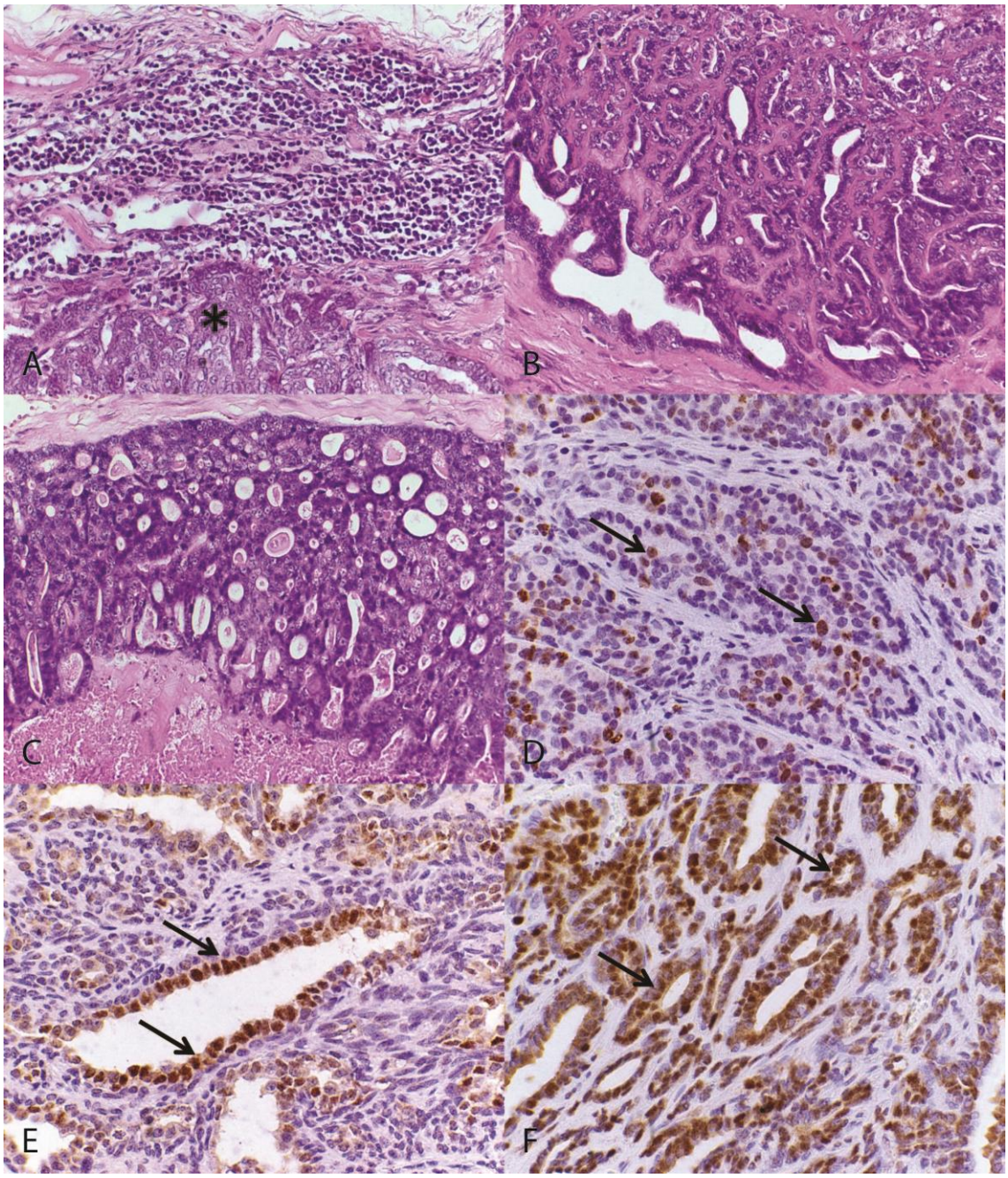
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Figure 1.

A. Feline lymph node. Regional metastasis composed by epithelial cells (asterisk). HE, 40x.

B. Feline mammary gland. Tubulopapillary carcinoma presenting an epithelial proliferation in a tubular and papillary pattern. HE, 40x.

C. Feline mammary gland. Cribriform carcinoma presenting an epithelial proliferation in a cribriform pattern. HE, 40x.

D. Feline mammary gland. Tubulopapillary carcinoma presenting neoplastic Ki-67-immunoreactive epithelial cells stained in brown (nuclei) (arrows). Polymeric detection system anti-Ki-67, counterstained with Harris's hematoxylin, 60x.

E. Feline mammary gland. Tubulopapillary carcinoma presenting neoplastic ER-immunoreactive epithelial cells stained in brown (nuclei) (arrows). Polymeric detection system anti-ER, counterstained with Harris's hematoxylin, 60x.

F. Feline mammary gland. Tubulopapillary carcinoma presenting neoplastic PR-immunoreactive epithelial cells stained in brown (nuclei) (arrows). Polymeric detection system anti-ER, counterstained with Harris's hematoxylin, 60x.

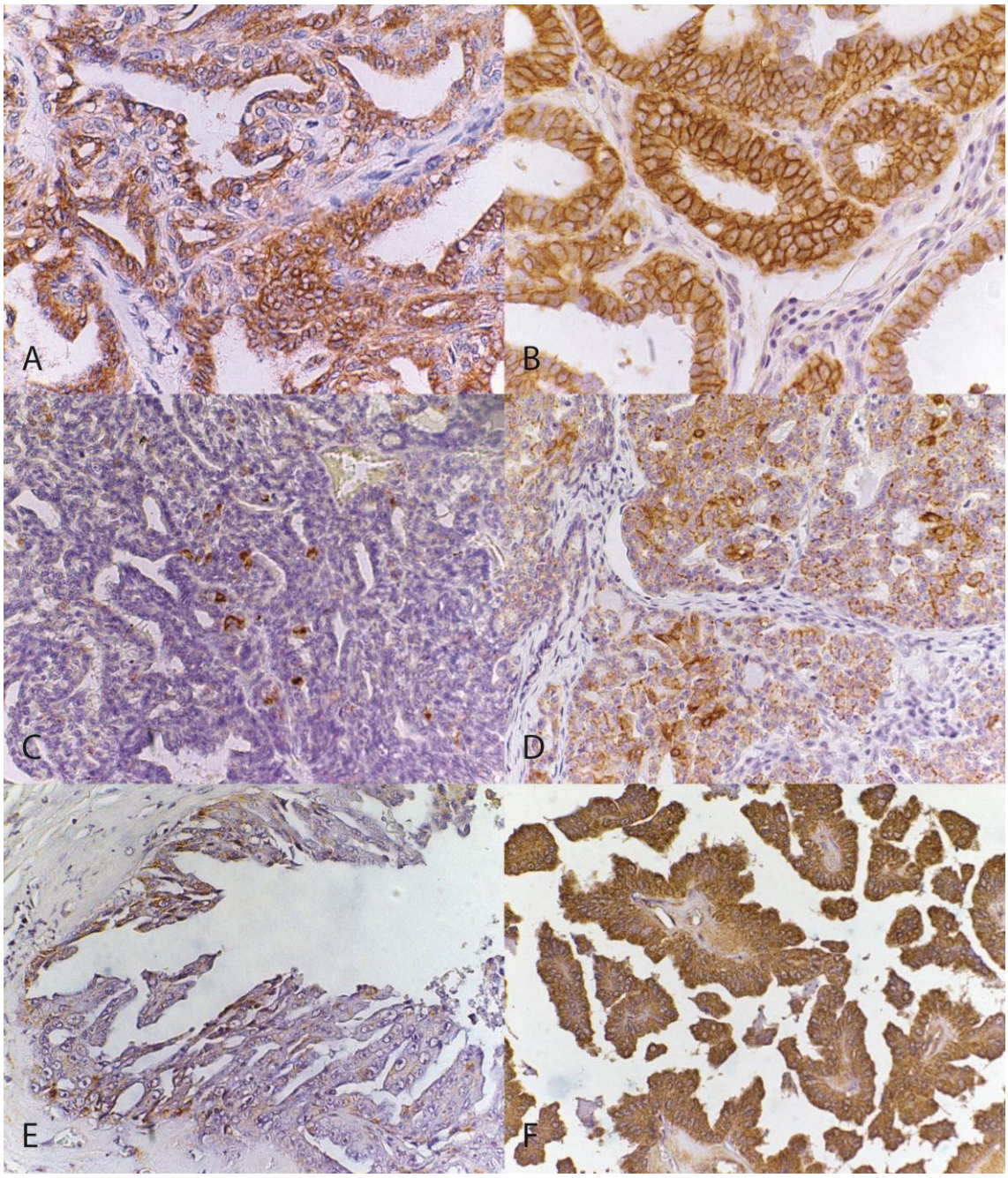
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Figure 2.

A. Feline mammary gland. Tubulopapillary carcinoma presenting neoplastic Her-2-immunoreactive score 1+ epithelial cells stained in brown (membrane). Polymeric detection system anti-Her-2, counterstained with Harris's hematoxylin, 40x.

B. Feline mammary gland. Tubulopapillary carcinoma presenting neoplastic Her-2-immunoreactive score 3+ epithelial cells stained in brown (membrane Polymeric detection system anti-Her-2, counterstained with Harris's hematoxylin, 40x.

C. Feline mammary gland. Tubulopapillary carcinoma presenting neoplastic low Cox-2-immunoreactive epithelial cells stained in brown (cytoplasm). Polymeric detection system anti-Cox-2, counterstained with Harris's hematoxylin, 40x.

D. Feline mammary gland. Tubulopapillary carcinoma presenting neoplastic high Cox-2-immunoreactive epithelial cells stained in brown (cytoplasm). Polymeric detection system anti-Cox-2, counterstained with Harris's hematoxylin, 40x.

E. Feline mammary gland. Tubulopapillary carcinoma presenting neoplastic VEGF-immunoreactive score 1 epithelial cells stained in brown (cytoplasm). Polymeric detection system anti-VEGF, counterstained with Harris's hematoxylin, 40x.

F. Feline mammary gland. Tubulopapillary carcinoma presenting neoplastic VEGF-immunoreactive score 3 epithelial cells stained in brown (cytoplasm). Polymeric detection system anti-VEGF, counterstained with Harris's hematoxylin, 40x.

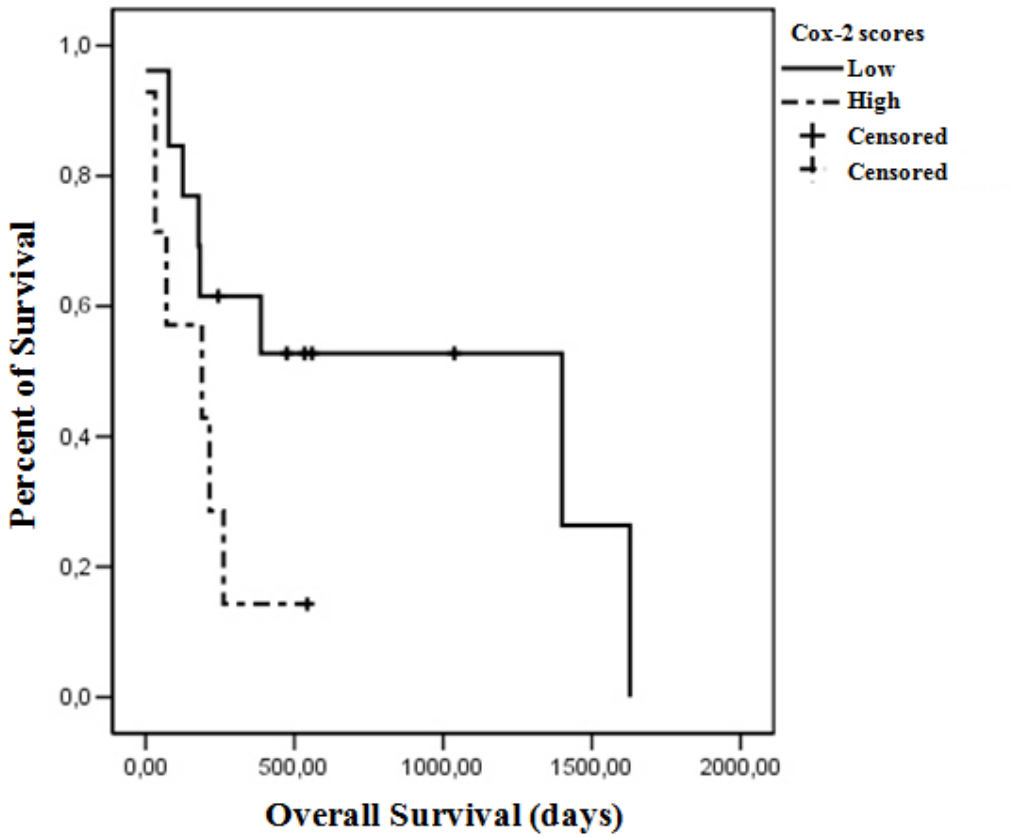

Figure 3. Overall survival curves for 20 female cats according to Cox-2 immunohistochemical staining. Queens with low Cox-2 score neoplasms, 13 cases; and high Cox-2 score neoplasms, 7 cases (p=0.089).

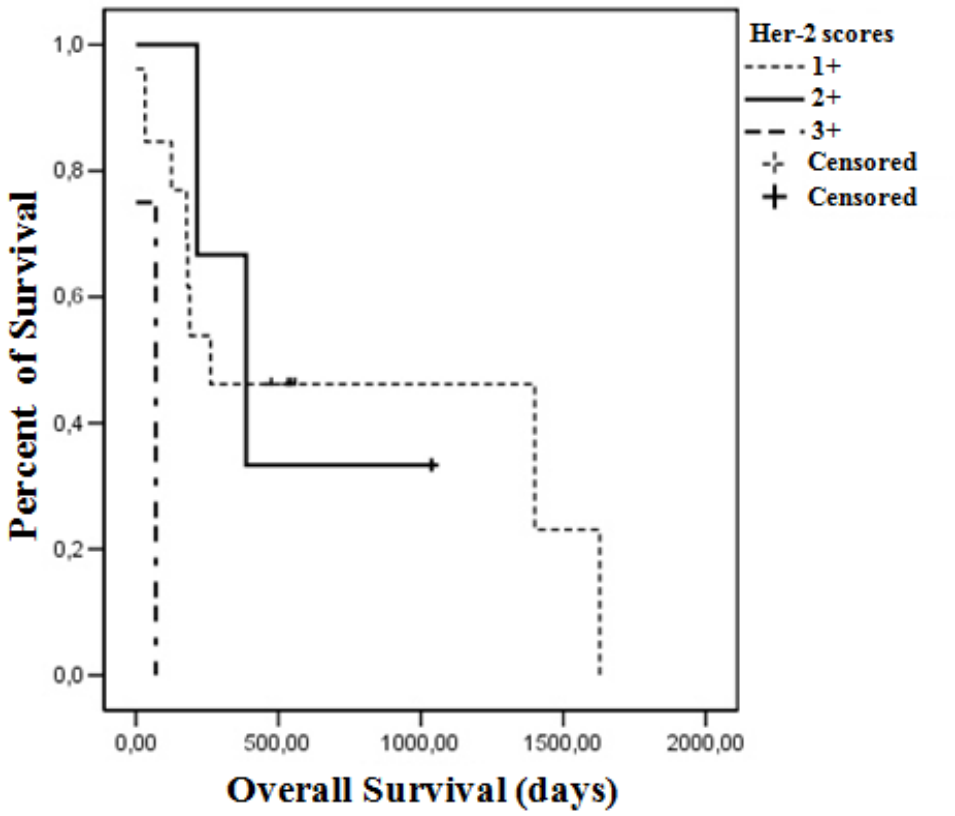

Figure 4. Overall survival curves for 18 female cats according to Her-2 immunohistochemical staining. Queens with Her-2 grade I neoplasms, 13 cases; Her-2 grade II neoplasms, 3 cases; Her-2 grade III neoplasms, 2 cases (p=0.012).

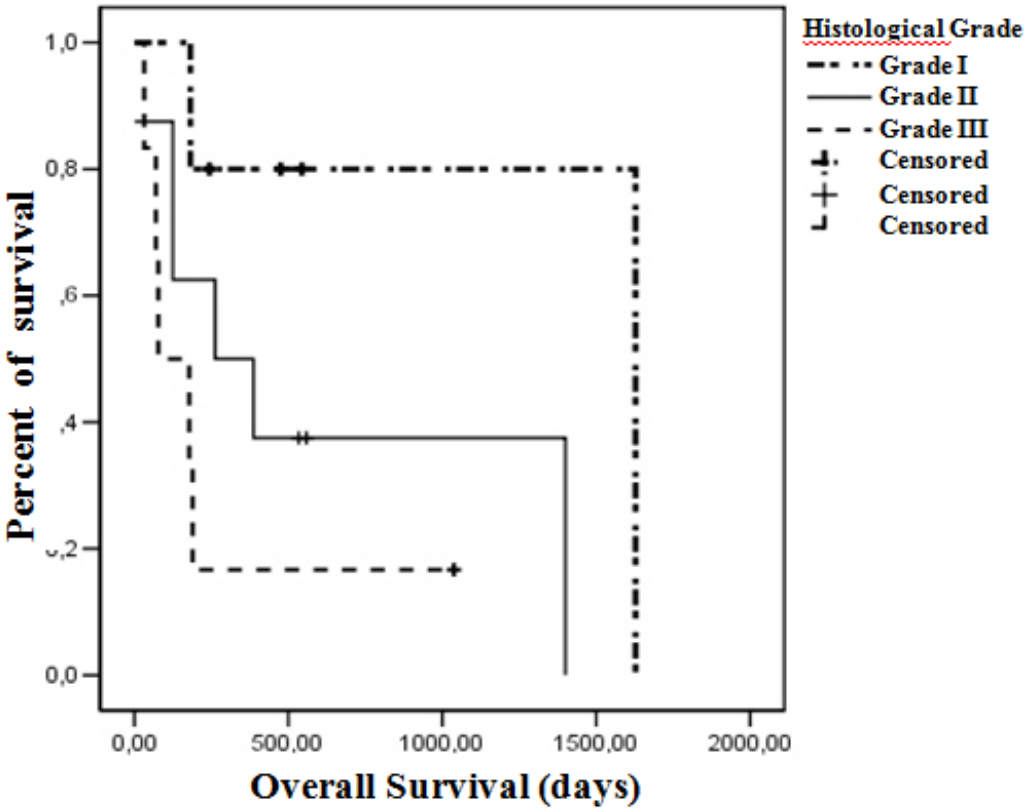

Figure 5. Overall survival curves for 19 female cats according to histological grade. Queens with grade I neoplasms, 5 cases; grade II neoplasms, 8 cases; grade III neoplasms, 6 cases (p=0.080).

ARTIGO 3

SPECIAL TYPES OF FELINE MAMMARY GLAND TUMORS: GLYCOGEN-RICH CLEAR CELL CARCINOMA AND MALIGNANT ADENOMYOEPITHELIOMA

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Abstract

Glycogen-rich clear cell carcinomas (GRCCC) are rare lesions, characterized by carcinoma cells containing an optically clear cytoplasm and intracytoplasmic glycogen. Malignant adenomyoepithelioma (MA) of the breast is also a rare tumor characterized by a malignant proliferation the luminal glandular component and myoepithelial cells. The aim of this paper was to report the first histopathological, immunohistochemical, and clinical characteristics of a MA and a GRCCC in the feline mammary gland. Both neoplasms presented similar histological features when compared to the human counterpart. Diagnosis was confirmed through PAS staining for GRCCC and immunohistochemical analysis of p63 for the MA. Knowledge of special types of tumors in veterinary medicine are important, especially when larger number of cases are studied and the prognostic of specific lesions are established, leading to appropriate treatment of patients.

Introduction

Mammary neoplasms are the third most frequent tumor that affects domestic felines (MACEWEN *et al.*, 1984; MISDORP, 2002; OVERLEY *et al.*, 2005; LANA *et al.*, 2007; GIMENÉZ *et al.*, 2010). Intact queens are more likely to develop the disease (LANA *et al.*, 2007), and average age of diagnosis is 10-11 years (MISDORP, 2002). Common characteristics include ulceration, lymphatic vessel invasion, and regional and distant metastasis (LANA *et al.*, 2007). Tubular, papillary, solid, cribriform, and *in situ* carcinomas are considered as common diagnosis in the feline mammary gland, and some carcinomas show a combination of histologic types (MISDORP *et al.*, 1999; MISDORP, 2002; LANA *et al.*, 2007).

In humans, glycogen-rich clear cell carcinomas (GRCCC) are characterized by carcinoma cells containing an optically clear cytoplasm and intracytoplasmic glycogen (MARTÍN-MARTÍN *et al.*, 2011). They are rare lesions, accounting for approximately 1-3% of all breast carcinomas. This type of tumor also arises in other organs including the lung, salivary gland, ovary, and endometrium (EUSEBI *et al.*, 2012; ROSEN, 2009a).

Adenomyoepithelioma of the human breast is also a rare tumor characterized by a proliferation of both the luminal glandular component as well as the myoepithelial cells. Most tumors are benign, but malignant transformation of one or both cellular components may

occur (AHMED; HELLER, 2000; HOWLETT *et al.*, 2003; JONES *et al.*, 2003; LAKHANI *et al.*, 2012; ROSEN, 2009b). Tumors derived from myoepithelial cells have been reported in the skin, salivary glands, breast, and lungs (GATTI *et al.*, 2004).

Although GRCCC and malignant adenomyoepithelilomas (MA) have been well described in the human mammary gland, these tumors have not been described in felines. Therefore, in this paper, we aim to report the first histopathological, immunohistochemical, and clinical characteristics of a glycogen-rich clear cell carcinoma and a malignant adenomyoepithelioma in the feline mammary gland.

Case Reports

Glycogen-rich clear cell carcinoma

A twelve year old neutered Persian queen presented painful abdominal masses, loss of appetite and weight loss. The patient underwent radical unilateral mastectomy. Overall survival was considered 33 days and death was due to clinical evolution of the disease.

Macroscopic evaluation of the resected sample demonstrated multiple focal masses in the cranial abdominal mammary gland of up to 0.5 cm in diameter, solid, not fixed, well demarcated and of whitish color. The caudal abdominal mammary gland presented a 5.5 x 3.8 x 1.2 cm ulcerated irregular mass of firm consistency and an isolated, 1.8 x 1.3 x 1.2 cm subcutaneous mass, also of firm consistency. Both masses were fixed, presented irregular invasive margins, solid and lobulated aspects, and a light yellow color. Axillary and inguinal enlarged lymph nodes were evaluated and presented characteristics similar to the mammary gland lesions, firm consistency, lobulated cut surface, fixed, invasive margins, solid, and whitish color.

Samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS). Microscopic evaluation demonstrated neoplastic masses characterized by an epithelial proliferation in a predominantly solid, with occasional tubular or papillary, arrangement with *in situ* and invasive areas. At least 75% of tumor cells presented an ample, granular, and foamy clear cytoplasm with central and round to ovoid nucleous and prominent nucleoli, cellular pleomorfism was considered moderate. PAS staining with diastase digestion revealed the

presence of citoplasmatic glycogen in neoplastic clear cells. Histological grade of primary tumors was considered as grade III, according to Elston and Ellis (1998). Lymph node histopathological evaluations demonstrated macrometastasis composed by epithelial cells. Metastatic cells were characterized by moderate pleomorfism with moderate cytoplasm, and multiple and prominent nucleoli. Extensive areas of necrosis were evidenced.

Histological sections of primary tumors and lymph nodes were obtained for immunohistochemical analysis; details are described in Table 1. Immunohistochemical procedures were identified using secondary antibodies (Advance HRP, DakoCytomation). Endogenous peroxidase activity was blocked with a solution of 3% H_2O_2 in methyl alcohol. Reagents were applied for 30 minutes manually and immunoreactivity was visualised by incubating the slides for 10 minutes with diaminobenzidine (DAB Substrate System; Dakocytomation). Results of immunohistochemical analysis are described in Table 2.

Histopathological and histochemical findings lead to the diagnosis of GRCCC with regional metastases to axillary and inguinal lymph nodes (Fig. 1 A and B).

Malignant adenomyoepithelioma

A fifteen year old neutered domestic shorthair queen presented mammary masses, with a 6 month evolution. Radical unilateral mastectomy with inguinal and axillary lymph node removal was performed. The proposed chemotherapy protocol started twenty days after surgery, with carboplatin at the dose of 200mg/m^2, with a 21 day interval. After two completed cycles, the patient presented local recurrence, clinical evolution of the disease, and death. Overall survival was considered 125 days.

Macroscopic evaluation of the resected samples demonstrated a 3.0x1.8x1.5 cm lobulated subcutaneous nodular formation between the caudal thoracic and cranial abdominal mammary glands. The cut surface presented a firm mass presenting coalescent nodules of approximately 1.0 cm with whitish color and cystic areas of approximately 0.2 cm diameter containing a brownish and vitreous material. The inguinal lymph node measured 2.0x1.5x1.0 cm, cut surface revealed loss of delimitation between the cortical and medullar regions, and a cystic central area of 0.6 cm.

Samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE). Microscopic evaluation of the primary neoplasm demonstrated a highly cellular malignant myoepithelial cell proliferation in a solid pattern, with moderate pleomorphism, loose chromatin, and prominent nucleoli. A malignant epithelial proliferation in tubular and papillary arrangement, presenting multiple *in situ* carcinomatous areas and areas of stromal invasion was also observed. Neoplastic epithelial cells were characterized by a moderate cytoplasm, moderate nuclear pleomorfism, and multiple and prominent nucleoli. Both components presented low mitotic index, areas of lymphatic invasion were evidenced and surgical margins were compromised. Histological grade was not obtained due to a predominance of the myoepithelial component and insufficient invasive epithelial areas. The inguinal lymph node presented macrometastasis in the cortical and medullar regions characterized by epithelial and myoepithelial cells, resembling the malignant adenomyoepithelioma. Epithelial cells were characterized by moderate nuclear pleomorfism, and multiple and prominent nucleoli. Myoepithelial cells were characterized by a solid pattern, with moderate pleomorphism, loose chromatin, and prominent nucleoli.

Immunohistochemical analysis was performed in primary tumors and lymph nodes as described above and the myoepithelial nature of neoplastic cells in primary tumor was confirmed by p63 3+ positivity. Other immunohistochemical results are also described in Table 2.

Based on histopathological and immunohistochemical findings the diagnosis of MA was determined (Fig. 1 C and D).

The patient presented another lymph node (axillary) metastasis and three mammary neoplasms that were removed during the surgical procedure. Cribriform carcinoma with tubular areas and lymphatic invasion, cribriform carcinoma with mucinous areas, and a tubulopapillary carcinoma were diagnosed. The axillary lymph node presented metastasis containing only epithelial cells, with a pattern resembling the cribriform carcinoma. Therefore, overall survival is not solely due to the reported neoplasm.

Table 1.: Target antigen, clone, manufacturer, dilution, antigen retrieval method, incubation time and temperature, and references used for analysis of immunohistochemical staining for Ki-67, Estrogen Receptor (ER), Progesterone Receptor (PR), Cyclooxigenase-2 (Cox-2), Vascular Endothelial Growth Factor (VEGF), Human Epidermal Growth Factor Receptor type 2 (Her-2), and p63.

Target Antigen (Clone)	Manufacturer	Dilution	Antigen Retrieval Method	Incubation Time (h) / Temperature	Analysis
Ki-67 (MIB-1)	Dakocytomation	1:25	Pressurised Heat (125°C/2min) with citrate buffer pH 6.0*	1 / Room Temperature	DUTRA *et al.*, 2004
RE (1D5)	Dakocytomation	1:20	Pressurised Heat (125°C/2min) with EDTA buffer pH 9.0*	1 / Room Temperature	HAMMOND *et al.*, 2010
RP (HPRA2)	Neomarkers	1:20	Pressurised Heat (125°C/2min) with EDTA buffer pH 9.0*	1 / Room Temperature	HAMMOND *et al.*, 2010
HER-2 (Polyclonal)	Dakocytomation	1:200	Water bath (98°C/20min) with citrate buffer pH 6.0*	16 / 4°C	WOLFF *et al.*, 2007
Cox-2 (SP21)	Neomarkers	1:80	Water bath (98°C/20min) with citrate buffer pH 6.0*	1 / Room Temperature	LAVALLE *et al.*, 2012
VEGF (Ab-1)	Neomarkers	1:200	No antigen retrieval	1 / Room Temperature	TSAI *et al.*, 2012
p63 (4A 4)	Neostains	1:80	Water bath (98°C/20min) with citrate buffer pH 6.0*	1 / Room Temperature	BERTAGNOLLI *et al.*, 2009

* DakoCytomation Target Retrieval Solution

Table 2. Immunohistochemical results for Ki-67, Estrogen Receptor (ER), Progesterone Receptor (PR), Cyclooxigenase-2 (Cox-2), Vascular Endothelial Growth Factor (VEGF), and Human Epidermal Growth Factor Receptor type 2 (HER-2) analysis in a glycogen-rich carcinomas and malignant adenomyoepithelioma.

Neoplasms			**Ki-67 (%)**	**ER**	**PR**	**Cox-2**	**VEGF**	**HER-2**
GRCCC	**Primary Neoplasms**	***Cranial abdominal***	43	+	+	6	2	1+
		Caudal Abdominal	64.6	+	+	8	3	1+
	Regional Lymph Nodes	**Axillary**	33.6	+	+	8	2	1+
		Inguinal	47.6	+	+	12	2	1+
MA	**Primary Tumor**		3	+	+	2	1	1+
	Lymph Node Metastasis		22	+	+	4	1	1+

Discussion

GRCCC of the breast is defined as a carcinoma in which more than 90% of tumor cells have abundant clear cytoplasm containing glycogen (EUSEBI *et al.*, 2012; ROSEN, 2009a). A special type of carcinoma is defined when the features compose more than 90% of the tumor, and when the special features are present in 75 to 90% of the tumor, it may be recognized as a variant (PAGE *et al.*, 1998). Therefore, the GRCCC in this study was considered a variant of this histological type. As was described in the present report, most human patients have lymph node metastasis at the time of clinical detection and tend to experience a less favorable outcome than those harboring non clear cell carcinomas (VARGA; CADUFF, 1999; SON *et al.*, 2004; ROSEN, 2009a). The diagnosis of this histological type is based on histologic and histochemical findings. GRCCC consist predominantly of cells containing PAS-positive, diastase-labile material (EUSEBI *et al.*, 2012; MIZUKAMI *et al.*, 2009; ROSEN, 2009a). Our results pointed to a poorly differentiated, high Ki-67 proliferation index, high Cox-2 scores, and VEGF overexpression. All of these findings are characteristic of poor prognostic in feline mammary neoplasms (MILLANTA *et al.*, 2002a; MILLANTA *et al.* 2002b; DIAS PEREIRA *et al.* 2004; MILLANTA *et al.* 2006; RASOTTO *et al.*, 2011; SEIXAS *et al.*, 2011), and are

compatible with the poor prognosis that this tumor type has on human breast cancer (EUSEBI *et al.*, 2012; ROSEN, 2009a; VARGA; CADUFF, 1999). Our PR results contradict human breast GRCCC, in which all described tumors were negative for this hormonal receptor. ER is described as positive in 50% of human cases (EUSEBI *et al.*, 2012; ROSEN, 2009a) and was positive in case reports by Markopoulos *et al. (*2008) and Mizukami *et al.* (2009), and negative by Son *et al.* (2004) and Martin-martin *et al.* (2011). As found in our study, Her-2 scores were negative in previous studies in humans (SON *et al.*, 2004; MARKOPOULOS *et al.*, 2008; MIZUKAMI *et al.*, 2009).

In humans, MA with a biphasic growth pattern at the primary site and in metastasis has been reported (ROSEN, 2009b), such as the present report. The epithelial component may form solid nests or groups, ducts, cystic trabecular, pseudo-papillary, or papillary structures. The myoepithelial component is arranged around the epithelial component and can form solid strands, trabeculae or even larger sheets, and is usually polygonal or spindle shaped (JONES *et al.*, 2003). In the present report, the epithelial component formed tubular and papillary structures, while the myoepithelial component had a solid arrangement, similar to the description of larger sheets. A clear diagnosis is enabled through immunohistochemical markers for myoepithelial cells as well as tumors derived from myoepithelial cells, and p63 is considered especially helpful (LAKHANI *et al.*, 2012; ROSEN, 2009b). Our results pointed to an ER and PR positive, lack of Her-2 and VEGF overexpression, low Ki-67 index, and low Cox-2 score in the primary tumor, all characteristic of a better prognosis in feline mammary gland neoplasms (MILLANTA *et al.*, 2002a; MILLANTA *et al.*, 2002b; DIAS PEREIRA *et al.*, 2004; MILLANTA *et al.*, 2005a; MILLANTA *et al.*, 2005b; MILLANTA *et al.*, 2006; RASOTTO *et al.*, 2011; SEIXAS *et al.*, 2011). The lymph node metastasis presented higher proliferation indexes and Cox-2 scores, indicating a selection of more aggressive neoplastic cells of primary tumor that metastasized. Metastases of adenomyoepitheliomas with one or two malignant components generally occurs in 40%, mostly to the lungs, but may also involve liver, bone, brain, and other sites. Regional lymph node metastasis, as was reported the queen, is unusual (LAKHANI *et al.*, 2012).

Features of GRCCC and MA in queens seem to be similar to the same tumors diagnosed in women, which enabled an adequate diagnosis. Knowledge of special types of tumors in

veterinary medicine are important, especially when larger number of cases are studied and the prognostic of specific lesions are established, leading to appropriate treatment of patients.

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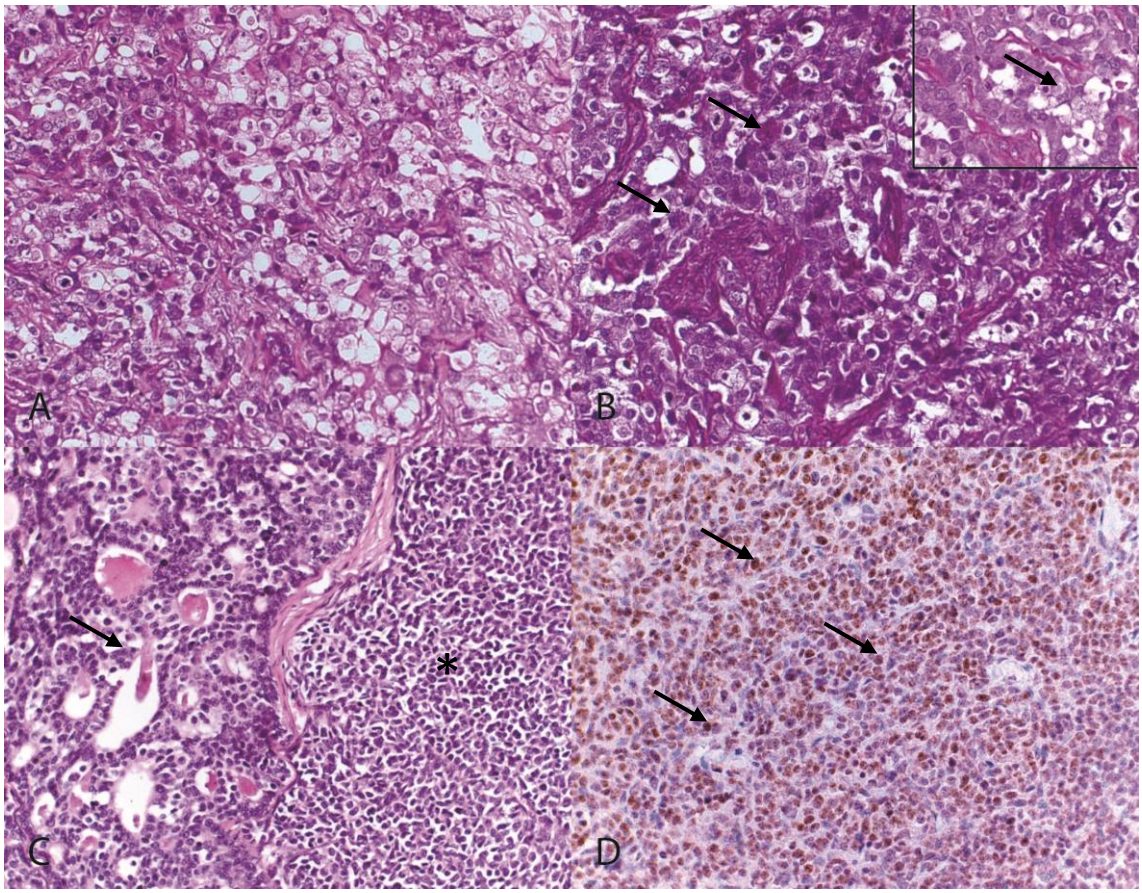

Figure 1. Photomicrographs illustrating features of glycogen-rich clear cell carcinoma (A and B) and malignant myoepithelioma (C and D) of the feline mammary gland: A. Tumor cells presenting moderate cellular pleomorphism an ample, granular, and foamy clear cytoplasm with central and round to ovoid nucleous and prominent nucleoli. HE, 40x. B. Neoplastic epithelial clear cells presenting cytoplasmatic PAS-positive glycogen (Inset shows of PAS staining after diastase digestion). PAS, 40x. C. Malignant neoplastic epithelial (Arrow) and myoepithelial cells (asterisk). HE, stain, 40x. D. Neoplastic epithelial cells presenting nuclear positivity for p63 antigen (Arrow) (Polymeric detection system anti-p63, counterstained with Harris's haematoxylin, 40x.

8. CONCLUSÕES FINAIS

A partir dos resultados obtidos nesse estudo, nas condições metodológicas empregadas, podemos concluir que:

- O benefício terapêutico da carboplatina associada ao tratamento cirúrgico permanece invalidado para carcinomas mamários em felino, não apresentando diferença na sobrevida global comparando ao tratamento cirúrgico de forma isolada;

- O estudo dos fatores prognósticos revelou que a inibição de hormônios ovarianos e ciclooxigenase-2 podem possivelmente representar uma opção terapêutica para neoplasias mamárias malignas felinas;

- A sobrevida global das gatas portadoras de neoplasias mamárias malignas foi diretamente influenciada pelo grau histológico, escore de Her-2 e escore de Cox-2;

- Escores de Cox-2 e Ki-67 foram maiores nas metástases regionais em relação aos tumores primários.

9. CONSIDERAÇÕES FINAIS

As neoplasias mamárias em felinos representam um desafio ao clínico por apresentarem um prognóstico reservado a desfavorável e sobrevida média inferior a um ano. Assim, torna-se necessário a definição de novas abordagens terapêuticas, assim como o conhecimento mais aprofundado da doença.

A literatura disponível relacionada com neoplasias mamárias em felinos ainda pode ser considerada escassa. Outros problemas também são encontrados. Trabalhos relacionados com tratamentos para essa doença estudam um número pequeno de animais, em caráter retrospectivo e com metodologias passíveis de críticas. Logo, mesmo trabalhos com resultados positivos podem ter sua validade questionada. Além disso, trabalhos mais direcionados para características patológicas das neoplasias mamárias em felinos em geral apresentam um grave problema de falta de padronização de metodologias empregadas. Assim, diferentes trabalhos abordando um mesmo tema não podem ser somados, pois resultados variam muito em função da metodologia utilizada. A falta de critérios diagnósticos estabelecidos para lesões mamárias em felinos tornou possível a descrição, pela primeira vez, de dois tipos histológicos em nossa pequena amostragem, cujos comportamentos e tratamentos ainda deverão ser estudados.

Nesse aspecto, podemos utilizar critérios já estabelecidos na medicina humana. Neoplasias mamárias em felinos são consideradas, por diversos autores, modelos para o estudo de neoplasias humanas devido à semelhança de varias características clinicam e patológicas. Podemos assumir então que o caminho inverso também pode ser realizado. Muitas metodologias em medicina humana são bem estabelecidas após estudos que comprovam sua eficiência. A possibilidade de padronização metodológica na medicina veterinária permitiria um crescimento significativo do conhecimento das doenças que acometem nossos pacientes.

Além disso, provavelmente devido à procura reduzida de atendimento veterinário por proprietários de gatos em centros de referência, o número pequeno de animais em cada estudo pode ser contornado com a colaboração de várias instituições. Estudos grandes multicêntricos podem possivelmente permitir maior número amostral e consequentemente, maior poder estatístico ao estudo.

Dentre os resultados obtidos no presente trabalho, alguns fatores prognósticos demonstraram potencial como fatores preditivos, podendo ser considerados como possíveis novas perspectivas terapêuticas. Essas novas perspectivas são desejáveis, considerando que nossos resultados não demonstraram o efeito benéfico da quimioterapia adjuvante com carboplatina na sobrevida global dos animais.

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11. APÊNDICES

APÊNDICE A. PRODUÇÃO CIENTÍFICA RELACIONADA À DISSERTAÇÃO NO PERÍODO DE MARÇO DE 2011 A MARÇO DE 2013

RESUMOS APRESENTADOS EM CONGRESSOS

- CAMPOS, C. B.; DAMASCENO, K. A.; GAMBA, C. O.; RIBERIRO, A. M.; LAVALLE, G. E.; CASSALI, G. D. Avaliação de fatores prognósticos e tratamento quimioterápico adjuvante em tumores mamários felinos. *III Encontro de Patologia*, Belo Horizonte, 2012.

APÊNDICE B. PRODUÇÃO CIENTÍFICA NÃO RELACIONADA À DISSERTAÇÃO NO PERÍODO DE MARÇO DE 2011 A MARÇO DE 2013

RESUMOS APRESENTADOS EM CONGRESSOS

- SILVA, J. O.; CAMPOS, L. C.; CAMPOS, C. B.; LAVALLE, G. E.; CASSALI, G. D. Avaliação sérica de LDH em cadelas com câncer de mama em diferentes estadios. *III Encontro de Patologia*, Belo Horizonte, 2012.

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12. ANEXOS

ANEXO A. Certificado do Comitê de Ética em Experimentação Animal – CETEA

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL
- C E T E A -

CERTIFICADO

Certificamos que o **Protocolo nº 13412/2012,** relativo ao projeto intitulado "***Avaliação de fatores prognósticos e tratamento quimioterápico adjuvante em tumores mamários felinos***", que tem como responsável(is) **Geovanni Dantas Cassali** , está(ão) de acordo com os Princípios Éticos da Experimentação Animal, adotados pelo ***Comitê de Ética em Experimentação Animal*** **(CETEA/UFMG),** tendo sido aprovado na reunião de **23/ 05/2012.**

Este certificado expira-se em **23/ 05/ 2017.**

CERTIFICATE

We hereby certify that the **Protocol nº 13412/2012,** related to the project entitled "***Evaluation of prognostic factors and adjuvant chemotherapy treatment in feline mammary tumors***", under the supervisiors of **Geovanni Dantas Cassali,** is in agreement with the Ethical Principles in Animal Experimentation, adopted by the ***Ethics Committee in Animal Experimentation*** **(CETEA/UFMG),** and was approved in **May 23, 2012.**

This certificate expires in **May 23, 2017.**

Belo Horizonte, 25 de Maio de 2012.

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ANEXO B. Comprovação da submissão de artigo científico para a revista Journal of Veterinary Internal Medicine

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25-Jan-2013

Dear Dr. Cassali:

Your manuscript entitled "USE OF CARBOPLATIN IN FELINE MALIGNANT MAMMARY GLAND NEOPLASMS WITH ADVANCED CLINICAL STAGING" by Campos, Cecilia; Nunes, Fernanda; Lavalle, Gleidice; Cassali, Geovanni, has been successfully submitted online and is presently being given full consideration for publication in the Journal of Veterinary Internal Medicine.

Co-authors: Please contact the Editorial Office as soon as possible if you disagree with being listed as a co-author for this manuscript.

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Thank you for submitting your manuscript to the Journal of Veterinary Internal Medicine.

Sincerely,
Journal of Veterinary Internal Medicine Editorial Office

MANUSCRIPT CONFLICT OF INTEREST DETAIL:

Conflict of Interest (Yes/No): No
If yes:
Explanation if yes: