UNIVERSIDADE ESTADUAL PAULISTA – UNESP
CÂMPUS DE JABOTICABAL

Avaliação do infiltrado inflamatório do microambiente tumoral e sua relação com diferentes tipos histológicos de neoplasias mamárias caninas

Thiago Alves de Souza
Médico Veterinário

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA DISSERTAÇÃO: AVALIAÇÃO DO INFILTRADO INFLAMATÓRIO DO MICROAMBIENTE TUMORAL E SUA RELAÇÃO COM DIFERENTES TIPOS HISTOLÓGICOS DE NEOPLASIAS MAMÁRIAS CANINAS

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Jaboticabal, 20 de outubro de 2017

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Thiago Alves de Souza – Nascido em 02 de dezembro de 1987, no município de Jaboticabal, estado de São Paulo. Ingressou no curso de Medicina Veterinária da Universidade Federal de Uberlândia no ano de 2009. Durante a graduação, foi membro do Programa de Educação Tutorial (PET) Medicina Veterinária, por meio do qual desenvolveu diversas atividades de ensino, pesquisa e extensão. Obteve o título de médico veterinário em fevereiro de 2014. De janeiro de 2014 a dezembro de 2014 fez aprimoramento profissional em Clínica Cirúrgica de Cães e Gatos pela Universidade de Uberaba (Uniube), Minas Gerais. Em março de 2015 ingressou no mestrado em Medicina Veterinária, sub-área Patologia Animal oferecido pela Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual "Júlio de Mesquista Filho", UNESP Jaboticabal, sob a orientação do prof. Dr. Geovanni Dantas Cassali. Foi bolsista CAPES por 18 meses e obteve aprovação em sua dissertação no dia 20/10/2017 pela banca examinadora.

EPÍGRAFE

“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê.” (Arthur Schopenhauer)

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Sumário

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Certificamos que o Protocolo nº 12455/15 do trabalho de pesquisa intitulado **"Expressão das células T reguladoras em carcinomas mamários caninos"**, sob a responsabilidade do Dr. Geovanni Dantas Cassali, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 06 de julho de 2015.

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Avaliação do infiltrado inflamatório do microambiente tumoral e sua relação com diferentes tipos histológicos de neoplasias mamárias caninas

RESUMO: As neoplasias mamárias constituem os tumores com maior incidência em cadelas. Dentre os fatores que contribuem para o desenvolvimento desta e de outras neoplasias, o microambiente tumoral inflamatório desempenha um papel crucial. Vários estudos relataram papéis importantes para linfócitos, macrófagos, plasmócitos, neutrófilos, eosinófilos e mastócitos neste contexto. No presente estudo, o objetivo foi avaliar a densidade de células inflamatórias e área de fibrose tumoral e sua relação com tumores mamários caninos com diferentes características histológicas e clínicas (tumor misto benigno, carcinoma em tumor misto, carcinoma sólido e carcinoma tubular). A análise de células inflamatórias e área de fibrose tumoral foram realizadas por meio de técnicas histoquímicas, enquanto a identificação de linfócitos Tregs foram realizadas por imuno-histoquímica. A análise estatística das densidades de células inflamatórias e áreas fibrose tumoral e sua relação com os tipos histológicos revelou diferença significativa para as plasmócitos ($p = 0,035$), neutrófilos ($p = 0,0113$), macrófagos ($p = 0,0047$) e fibrose tumoral ($p = 0,05$). Os dados encontrados sugerem associações entre alto número de neutrófilos e neoplasias mamárias agressivas, entre altas densidades de plasmócitos, macrófagos e células $CD8^+$ e entre menor densidade de células $CD4^+$ e neoplasias menos agressivas. Maiores áreas de fibrose tumoral mostraram relação com neoplasias mamárias caninas mais agressivas.

Palavras-chave: câncer, cadelas, histoquímica, imuno-histoquímica, inflamação

Evaluation of the inflammatory infiltrate of the tumor microenvironment and its relation to different histologic types of canine mammary gland tumors

ABSTRACT: Mammary neoplasias constitute the tumors with higher incidence in bitches. Among the factors that contribute for the development of this and other neoplasias, the inflammatory tumor microenvironment plays a crucial role. Several studies reported important roles for lymphocytes, macrophages, plasma cells, neutrophils, eosinophils and mast cells in this context. In the present study, the goal was to evaluate density of inflammatory cells and area of tumor fibrosis and their relation with canine mammary tumors with different histologic and clinical presentation (benign mixed tumor, carcinoma in mixed tumor, solid carcinoma and tubular carcinoma) Counting and staining of inflammatory cells and tumor fibrosis were performed through histochemistry, while counting and staining of Tregs were performed through immunohistochemistry. Statistical analysis of the densities of inflammatory cells and tumor fibrosis related to histologic types revealed significant difference for plasma cells (p=0,035), neutrophils (p=0,0113), macrophages (p=0,0047), and tumor fibrosis (p=0,05). The found data suggest associations between high number of neutrophils and agressive mammary neoplasias, between high densities of plasma cells, macrophages and $CD8^+$ cells and between low density of $CD4^+$ cells and less aggressive neoplasias. Higher areas of tumor fibrosis showed relation with more aggressive canine mammary neoplasias.

Keywords: cancer, bitches, histochemistry, immunohistochemistry, inflammation

CAPÍTULO I – Considerações gerais

1. Introdução e revisão de literatura

1.1 Neoplasias mamárias caninas

De acordo com a definição proposta por Kusewitt (2013), as neoplasias são caracterizadas por crescimento de células derivadas de tecidos normais que sofreram alterações genéticas herdadas ou induzidas, as quais podem tornar o tumor não responsivo às defesas do sistema imune.

As neoplasias mamárias correspondem à cerca de 50% da ocorrência de todos os tumores nas cadelas (OLIVEIRA et al., 2003; OLIVEIRA FILHO et al., 2010), sendo consideradas o tipo tumoral mais comumente diagnosticado nas fêmeas desta espécie (QUEIROGA; LOPES, 2002; ZATLOUKAL; KOHOUT; REPUBLIC, 2005). São mais frequentes em cadelas de meia idade a idosas, sem predisposição racial e podem apresentar correlação com características reprodutivas, uso de progestágenos e episódios de pseudociese, como aponta o estudo de Oliveira et al. (2003).

De forma geral, as neoplasias mamárias compõem um grupo heterogêneo de neoplasias, que são classificadas em diversos tipos histológicos. Dentre os tipos histológicos abordados no presente estudo, os Tumores Mistos Benignos e os Carcinomas em Tumores Mistos são conhecidos por apresentarem um melhor prognóstico frente aos tratamentos preconizados. Já o Carcinoma Sólido e o Carcinoma Tubular de graus mais elevados são tipos histológicos que geralmente apresentam pior prognóstico e são consideradas, portanto, como neoplasias mais agressivas (CASSALI et al., 2014).

Os tumores mistos são neoplasias que exibem um padrão histológico complexo, pois apresentam elementos do epitélio e do mesênquima, além de possuírem capacidade de passar por transformação maligna, originando principalmente carcinomas em tumores mistos e, menos frequentemente, carcinossarcomas e sarcomas em tumores mistos. Os tumores mistos benignos são caracterizados pela presença de elementos epiteliais benignos, como células ductais, acinares e mioepiteliais e células mesenquimais com formação de cartilagem e/ou osso juntamente com tecido fibroso mixóide. Já os carcinomas em tumores mistos são caracterizados por um desenvolvimento focal ou nodular de malignidade em um tumor misto primariamente benigno. A proliferação carcinomatosa do carcinoma em tumor misto pode exibir crescimento in situ ou infiltrativo, sendo este indicado pela perda de

continuidade das camadas basal e mioepitelial associada com a invasão do estroma por células neoplásicas (CASSALI et al., 2012).

O carcinoma sólido é caracterizado pela proliferação de células epiteliais organizadas em um arranjo sólido, com formação de cordões, lençóis ou aglomerados sem a formação de lúmen. Os lóbulos são de formato irregular e sustentados por um estroma fibrovascular fino. As células tumorais são indiferenciadas, de formato poligonal a oval com citoplasma escasso e exibem núcleos pequenos e hipercromáticos. Anisocariose e anisocitose estão presentes de forma moderada a intensa e o índice de mitose geralmente é alto. A infiltração de vasos linfáticos por células neoplásicas é comum, bem como metástase para os linfonodos regionais (CASSALI, 2002; CASSALI et al., 2014; GOLDSCHMIDT et al., 2017).

O Carcinoma Tubular é uma neoplasia caracterizada por proliferação epitelial organizada em arranjos tubulares e pela quantidade variável de estroma. Apresenta pleomorfismo nuclear moderado, sendo que os núcleos podem ser hipocromáticos com marginalização da cromatina, eucromáticos ou hipercromáticos com cromatina aglutinada. Os nucléolos podem ser únicos e grandes ou múltiplos e pequenos. O estroma interlobular é composto por vasos e fibroblastos e pode apresentar infiltração de plasmócitos, linfócitos e macrófagos. Esses tumores tendem a infiltrar vasos e tecido mamário vizinho, onde possuem a capacidade de evocar uma resposta estromal com extensa proliferação de miofibroblastos (CASSALI, 2002; CASSALI et al., 2014; GOLDSCHMIDT et al., 2017).

1.2. As células inflamatórias no microambiente tumoral

A primeira evidência de conexão entre inflamação e câncer data do século XIX, quando Rudolf Wirchow observou a presença de leucócitos em tumores. No entanto, foi só na última década que surgiram evidências claras do papel da inflamação na tumorigênese. Sabe-se que diversos tipos de processos inflamatórios, diferentes quanto a causa, mecanismo, consequência e intensidade, podem promover desenvolvimento e progressão neoplásica (GRIVENNIKOV et al., 2010).

O microambiente tumoral inflamatório é caracterizado pela presença de células da imunidade inata (macrófagos, neutrófilos, mastócitos, células dendríticas e células assassinas naturais), células da imunidade adaptativa (linfócitos T e B), além de células tumorais e do estroma circundante (fibroblastos, células endoteliais, pericitos

e células mesenquimais) (DE VISSER et al., 2006). Abaixo estão descritas as características mais importantes das principais células inflamatórias associadas ao microambiente tumoral.

1.2.1 Linfócitos

Existem muitas populações distintas de linfócitos no infiltrado inflamatório do microambiente tumoral, destacando-se principalmente os linfócitos T citotóxicos, caracterizados pela presença do marcador $CD8^+$, os linfócitos Th1 e Th2, caracterizados pela presença do marcador $CD4^+$ (Fridman et al., 2012) e os linfócitos T reguladores (Tregs), caracterizados pelo imunofenótipo $CD4^+CD25^+FOXP3^+$ (HSIEH et al., 2012).

De forma geral, os linfócitos T citotóxicos são células de memória capazes de reconhecer os antígenos das células tumorais e eliminá-las, atuando a favor do organismo do hospedeiro. O aumento dessas células geralmente está associado a um bom prognóstico. A ação dos linfócitos $TCD8^+$ é complementada por linfócitos T $CD4^+$ Th1, que produzem citocinas, tais como interleucina-2 (IL-2) e interferon gamma (IFN-γ. O número aumentado destas células também pode indicar um bom prognóstico (FRIDMAN et al., 2012).

Miyashita et al. (2015), ao avaliarem os tumores residuais de 131 pacientes humanos com câncer de mama triplo negativo, previamente submetidos a quimioterapia neoadjuvante, verificaram que altas taxas de linfócitos $CD8^+$ estavam associadas a melhores índices de sobrevida e a maior tempo livre de recidiva, de forma similar a Sharma et al. (2007), que encontraram as mesmas associações em carcinomas uroteliais de humanos. No mesmo estudo, Miyashita et al. (2015) também verificaram altas taxas de CD8/FOXP3 relacionadas a maior índice de sobrevida e maior tempo livre de recidiva, com resultados similares aos descritos por Asano et al. (2016).

Nguyen et al. (2016) testaram, por meio de imunoterapia, linfócitos $TCD8^+$ provenientes de três fontes diferentes, classificadas como “naïve” (NTeff), memória (MTeff) e linfócitos infiltradores de tumores (TILeff) e verificaram que a linhagem NTeff foi refratária a apoptose induzida por TGF-β (esclarecer) e apresentou efeito citotóxico mais potente, sugerindo o uso de linfócitos T citotóxicos no tratamento de neoplasias.

Os estudos envolvendo a participação dos linfócitos T $CD4^+$ no desenvolvimento e progressão de neoplasias mostram resultados controversos.

Macchetti et al. (2006), por exemplo, demonstraram alta infiltração de linfócitos T $CD4^+$ em pacientes humanas portadoras de carcinoma mamário com metástase em linfonodo, de forma semelhante a Droeser et al. (2012), que verificaram, em pacientes humanas com carcinoma ductal de mama, números elevados de linfócitos T $CD4^+$ e $FOXP3^+$ associados a tumores mais agressivos.

No entanto, a presença dos linfócitos T $CD4^+$ Th2, que favorecem a ação dos linfócitos B por meio da produção de IL-4, IL-5 e IL-13, se mostrou associada à promoção do crescimento tumoral (FRIDMAN et al., 2012) e menor taxa de sobrevida em pacientes humanas com câncer de mama e ovário (MOHAMMED et al., 2013; LUNDGREN et al., 2016). Apesar desses dados, Yoon et al. (2011) encontraram associação entre a presença de plasmócitos e melhor prognóstico em câncer de mama humano, assim como Knief et al. (2016), que verificaram sobrevida prolongada em pacientes portadores de adenocarcinoma de transição esofagogástrica com alta densidade de plasmócitos e linfócitos B.

Ao avaliarem a densidade de linfócitos no infiltrado inflamatório de carcinomas mamários em humanos, Macchetti et al. (2006) verificaram que o aumento de células $CD4^+$ estava relacionado a um pior prognóstico e à presença de metástases, de forma semelhante aos estudos de Estrela-Lima et al. (2010) e Carvalho et al. (2016), que verificaram relação semelhante em carcinomas mamários caninos e humanos. Com base nos resultados encontrados, Estrela-Lima et al. (2010) apontam a possibilidade de muitos desses linfócitos $CD4^+$ serem T reguladores (Tregs), principalmente quando associados à diminuição concomitante de linfócitos $CD8^+$, sugerindo importante participação da imunotolerância no desenvolvimento de carcinomas mamários caninos.

Os linfócitoTregs compõem um grupo de células responsáveis pela tolerância e homeostasia do organismo (MARTIN et al., 2010) e desempenham papel fundamental no combate a síndromes autoimunes, como mostram os estudos de Takahashi et al. (1998) e Fontenot et al. (2003). Estas células são divididas em linfócitos Treg de desenvolvimento natural no timo (nTreg) e linfócitos Treg adaptativos ou periféricos. Os nTregs são responsáveis pela resposta a antígenos próprios (SAKAGUCHI et al., 2008) e caracterizados pelo imunofenótipo T $CD4^+CD25^+FOXP3^+$. Os iTregs são responsáveis pela resposta a antígenos não próprios (THOMPSON, 2011) e caracterizados pela expressão de interleucina-10 (IL-10) e fator de crescimento tumoral β (TGF- β) (JONULEIT et al., 2002).

A ação das células nTreg na manutenção da tolerância a antígenos próprios constitui um dos mecanismos responsáveis por prejudicar a imunovigilância contra células tumorais autólogas (DUNN et al., 2004). Diversos estudos foram conduzidos com o intuito de tentar associar a expressão das células T $CD4^+$ $CD25^+$ $FOXP3^+$ a neoplasias mais agressivas, à presença de metástases e, consequentemente, a um pior prognóstico. O estudo de Liyanage et al. (2002) é um exemplo, onde foi demonstrado em mulheres, um número elevado de linfócitos Tregs em pacientes com câncer de mama (16,6%) e de pâncreas (13,2%) em relação às pacientes saudáveis (8,6%).

Bates et al. (2006) analisaram a expressão de FOXP3 em mamas de mulheres portadoras de carcinoma ductal *in situ* e carcinoma invasivo, contrapondo a um grupo formado apenas por tecido mamário normal e concluíram que o número de linfócitos Tregs foi maior nas mamas neopláiscas, especialmente nos tumores de alto grau, nas pacientes com acometimento de linfonodos regionais e nos tumores negativos para receptores de estrógeno (RE). Já no estudo de Bohling e Allison (2008) foram observadas associações entre o número elevado de linfócitos $FOXP3^+$ e tumores mamários mais agressivos de grau avançado na escala de Nottingham (grau III), tumores de maior tamanho e tumores RE negativos. Além disso, um dos fenótipos mais agressivos de câncer de mama humano, chamado de triplo negativo (Receptor de Estrógeno, Receptor de Progesterona e marcador HER2/neu negativos) também foi associado a números elevados de células FOXP3 positivas. Isso sugere a presença de maior imunotolerância em tumores mais agressivos.

Na Medicina Veterinária, O'Neil et al. (2009) avaliaram, por meio da citometria de fluxo, a presença de células Tregs tanto em cães saudáveis, quanto em cães portadores de carcinomas, sarcomas, linfoma e mastocitoma. Os autores verificaram um aumento na porcentagem de células $CD4^+$ $FOXP3^+$ nos animais doentes quando comparados aos animais sadios, principalmente naqueles que apresentavam carcinomas. Além disso, também foram avaliados os números de células $CD4^+$, $CD8^+$ e foi observado um aumento na relação Treg/$CD8^+$ nos animais com câncer, destacando ação supressora das células Treg.

1.2.2 Neutrófilos

Em relação à ação dos neutrófilos associados a tumores, estudos prévios demonstraram que essas células desempenham papéis pró-tumorigênicos, como estímulo da angiogênese (NOZAWA et al., 2006), aumento da degradação da matriz extracelular (DE LARCO et al., 2004) e imunossupressão (YOUN e GABRILOVICH, 2010) por meio do imunofenótipo N2 (GRANOT; JABLONSKA, 2015). Por outro lado, estudos mostram que essas células também são capazes de exercer atividade antitumoral por meio de citotoxicidade direta (DISSEMOND et al., 2003), citotoxicidade de anticorpo dependente de mediação celular (GUETTINGER et al., 2010; HUBERT et al., 2011) e estimulação de células T efetoras (FRIDLENDER et al., 2010), atividades características do fenótipo N1 (GRANOT; JABLONSKA, 2015).

1.2.3 Macrófagos

Os macrófagos são um dos principais componentes na conexão entre inflamação e câncer e uma fonte importante de citocinas. De forma semelhante aos linfócitos T, os macrófagos passam por processo de polarização, adquirindo fenótipo M1 ou M2. O que difere os macrófagos polarizados são a expressão de receptores, função de citocinas efetoras e produção de quimiocinas.

De forma geral, os macrófagos M1 são caracterizados por altos níveis de óxido nítrico-sintase induzida (iNOS), produção de IL-12 e fator de necrose tumoral (TNF), enquanto os macrófagos M2 são caracterizados pela produção de ornitina e poliaminas, IL-10, receptor antagonista de IL-1 (IL-1ra) e receptor decoy de IL-1 tipo II. (MANTOVANI et al., 2002; 2008).

Como já evidenciado por DiNapoli et al. (1996), os macrófagos associados a tumores (MATs) possuem um fenótipo semelhante aos macrófagos M2, já que apresentam baixa toxicidade para células tumorais, baixa produção de óxido nítrico, alta expressão de IL-10 e baixa expressão de IL-12.

Ao avaliarem os efeitos dos macrófagos diferentes neoplasias, Qian e Pollard (2010) relataram efeito pro-tumorigênico em modelos experimentais, de forma semelhante a Bingle et al. (2002), que observaram pior prognóstico nos tumores cujo microambiente tumoral mostrava abundância desse tipo celular. No entanto, apesar de menos frequente, há evidências de participação dos macrófagos M1 no ambiente tumoral, como nos carcinomas pulmonares de grandes células, apontado por Almatroodi et al. (2016).

1.2.4 Eosinófilos

Existem evidências de que, no ambiente tumoral, o papel do eosinófilo se dê de forma indireta por meio da liberação de mediadores responsáveis pelo recrutamento de células T $CD8^+$ e assassinas naturais (NK) (FURBERT-HARRIS et al., 2003; CARRETERO et al., 2015), exercendo, portanto, uma ação antitumoral e protetora. Amini et al. (2007) demonstraram que, nos carcinomas mamários invasivos, os eosinófilos não se apresentam em número significativo.

1.2.5 Mastócitos

A presença e atividade dos mastócitos no microambiente tumoral ainda permanece controversa. Alguns autores como Cimpean et al. (2017) e Lavalle et al. (2010) demonstraram que a densidade de mastócitos no microambiente tumoral de carcinomas mamários de mulheres e cadelas, estava relacionada a maior liberação de fatores angiogênicos e, consequentemente, à densidade de microvasculatura. Por outro lado, em carcinomas mamários humanos, foi observada alta densidade de mastócitos relacionada a tumores de baixo grau e positivos para receptores de estrógeno, a pacientes sem acometimento de linfonodo e a pacientes com melhor prognóstico (DABIRI et al., 2004; AMINI et al., 2007; RAJPUT et al., 2008).

1.3 Fibrose tumoral (incluir trabalho sobre neoplasias mamárias caninas)

Junto à ação das células inflamatórias, o estroma, tanto intratumoral quanto peritumoral, desempenha um papel importante na progressão e desenvolvimento das neoplasias, já que exibe componentes pró-tumorigênicos, como fibroblastos, células endoteliais, pericitos e células mesenquimais (DE VISSER et al., 2006). Recentemente, De Kruijf et al (2011) e Gujam et. al (2014) demonstraram a importância prognóstica da fibrose tumoral em neoplasias mamárias de mulheres, onde foi verificada que maior área de fibrose estava relacionada a menor tempo de sobrevida e menor tempo livre de recidiva. De forma semelhante, Liu et al. (2014) e Zhang et al. (2015) verificaram relação semelhante em carcinoma cervical e carcinoma pulmonar de grandes células, respectivamente.

Apesar de a literatura apresentar vários estudos abordando as relações entre células inflamatórias, fibrose tumoral e neoplasias mamárias, esses dados se referem, em maior parte, a pacientes humanas. Portanto, é necessário que se realizem mais

estudos relacionando esses parâmetros em neoplasias mamárias caninas, de forma a esclarecer como o microambiente inflamatório tumoral se comporta nessa espécie.

2. Objetivos

Tomando essas informações como base, o presente estudo foi realizado sob o objetivo de verificar a relação entre área de fibrose tumoral e a densidade de células inflamatórias com tipo histológico e a sobrevida de cadelas com neoplasias mamárias.

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CHAPTER 2 - Relationship between the inflammatory tumor microenvironment and different histologic types of canine mammary tumors

Relationship between the inflammatory tumor microenvironment and different histologic types of canine mammary tumors

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ABSTRACT: Mammary neoplasias are the tumors with higher incidence in bitches. Among the factors that contribute for the development of this and other neoplasias, the inflammatory tumor microenvironment plays a crucial role. Several studies reported important roles for lymphocytes, macrophages, plasma cells, neutrophils, eosinophils and mast cells in this context. In the present study, the aim was to evaluate density of inflammatory cells and area of tumor fibrosis and their relation with canine mammary tumors with different histologic and clinical presentation (benign mixed tumor, carcinoma in mixed tumor, solid carcinoma and tubular carcinoma) Counting and staining of inflammatory cells and tumor fibrosis were performed through histochemistry, while counting and staining of Tregs were performed through immunohistochemistry. Statistical analysis of the densities of inflammatory cells and tumor fibrosis related to histologic types revealed significant difference for plasma cells (p=0,035), neutrophils (p=0,0113), macrophages (p=0,0047), and tumor fibrosis (p=0,05). The found data suggest associations between high number of neutrophils and agressive mammary neoplasia, between high densities of plasma cells,

macrophages and $CD8^+$ cells and between low density of $CD4^+$ cells and less aggressive neoplasias. Higher areas of tumor fibrosis showed relation to more aggressive canine mammary neoplasias.

Keywords: cancer, bitches, histochemistry, immunohistochemistry, inflammation

1. Introduction

Mammary neoplasms represent approximately 50% of the tumors occurrence in bitches. These tumors are more frequent in middle-aged and elderly bitches, with no breed predisposition, and may show correlation with reproductive status, use of progestogen and episodes of pseudocyesis (OLIVEIRA et al., 2003).

In addition to the characteristics aforementioned, one of the factors that play an important role in tumor development is the inflammatory microenvironment (MANTOVANI et al., 2008). It is known that several types of inflammatory processes, different as to cause, mechanism, consequence and intensity, can promote neoplastic development and progression (GRIVENNIKOV et al., 2010). Based on recent studies, the cell types that have most been addressed in this microenvironment are T and B lymphocytes, macrophages and neutrophils (BALKWILL et al., 2012).

Among different populations of T lymphocytes, cytotoxic T lymphocytes are characterized mainly by the presence of CD8 marker and are generally associated with better prognosis (MAHMOUD et al., 2011; PRESTON et al., 2013; MIYASHITA et al., 2015). However, Th2 lymphocytes, characterized by the presence of CD4 marker (FRIDMAN et al., 2012) are related to worse prognostics (MACCHETTI et al., 2006). Regulatory T lymphocytes (Treg) are characterized by the immunophenotype $CD4^+$ $CD25^+$ $FOXP3^+$ and are associated with worse prognosis and lower survival (HSIEH; LEE; IOL, 2012).

B lymphocytes and plasma cells are associated with tumor growth promotion (Fridman et al., 2012) and lower survival rates in human patients with breast and ovarian cancer (Mohammed et al., 2013; Lundgren et al., 2016). However, Yoon et al. (2011) and Knief et al. (2016) reported a relationship between the increase in plasma cells and B lymphocytes numbers, breast cancers with better prognosis and prolonged survival in patients with esophagogastric transition adenocarcinoma.

Tumor-associated neutrophils showed pro-tumorigenic roles through angiogenesis stimulation (NOZAWA et al., 2006), increased degradation of

extracellular matrix (DE LARCO et al., 2004) and immunosuppression (YOUN and GABRILOVICH, 2010). These cells also exert antitumor activity through direct cytotoxicity (DISSEMOND et al., 2003), cell-mediated cytotoxicity (GUETTINGER et al., 2010; HUBERT et al., 2011) and stimulation of effector T cells (FRIDLENDER et al., 2010)

Regarding the macrophages, Qian and Pollard (2010) reported pro-tumorigenic effect of these cells in experimental models of cancer, similar to Bingle et al. (2002), who observed worse prognosis in tumors that showed abundance of this cell type. However, the increase of macrophages in the tumor microenvironment may also be associated with better prognosis and prolonged survival in large cell lung carcinomas (ALMATROODI et al., 2016)

The role of eosinophils in the tumor microenvironment is determined by the release of mediators that recruit $CD8^+$ T cells and natural killers cells (NK) (FURBERT-HARRIS et al., 2003; CARRETERO et al., 2015), guaranteeing antitumor and protective activities. On the other hand, Amini et al. (2007) showed that eosinophils are not present in significant number in invasive mammary carcinomas, making it impossible to elucidate the role of these cells in these types of neoplasms.

The presence and activity of mast cells in the tumor microenvironment remain controversial. Lavalle et al. (2010) and Cimpean et al. (2017) verified that high density of mast cells in tumor microenvironment was related to greater release of angiogenic factors and high density of microvasculature in canine and human mammary carcinomas, respectively. On the other hand, Dabiri et al. (2004), Amini et al. (2007), and Rajput et al. (2008) observed, in human mammary carcinomas, that a high density of mast cells was related to low grade tumors and estrogen receptor positivity, to patients without lymph node involvement and to patients with better prognosis, respectively.

In addition to the roles of inflammatory cells, De Kruijf et al. (2011) and Gujam et al. (2014) demonstrated a prognostic importance of tumor fibrosis in human mammary neoplasias by verifying relationship between higher areas of tumor fibrosis, survival time and relapse-free time. Furthermore, Liu et al. (2014) and Zhang et al. (2015) found a similar relationship in cervical carcinoma and large cell lung carcinoma in humans, respectively.

Although the current literature shows several studies that reports the participation of inflammatory cells and tumor fibrosis in tumor development and

progression in human species, the canine species lacks data regarding the role of inflammatory tumor microenvironment in the same context.

The present study was carried out to evaluate the densities of inflammatory cells and tumor fibrosis percentage in mammary neoplasms and relate these parameters to four different histologic types of these neoplasias in canine species.

2. Material and methods

Samples of mammary neoplasms from 38 bitches were selected from paraffin blocks of the Comparative Pathology Laboratory - Biological Sciences Institute of the Federal University of Minas Gerais (UFMG, Belo Horizonte, MG). Mammary neoplasms were classified according to the World Health Organization (MISDORP et al., 1999) and the guidelines of the second consensus for diagnosis, prognosis and treatment of canine mammary tumors (CASSALI et al., 2014)

Samples were divided in benign mixed tumor (BMT, n = 7), carcinoma in mixed tumor (CMT, n = 16), solid carcinoma (SC, n = 8) and tubular carcinoma (TC). Paraffin blocks were cut to a thickness of 3µm and, subsequently, either stained with hematoxylin and eosin for tumor classification, identification of micro invasion or invasion areas and counting of lymphocyte, plasma cells and macrophages under light microscopy, or submitted to histochemistry and immunohistochemistry techniques for counting of mast cells, eosinophils and tumor fibrosis percentage.

For immunohistochemistry, cuts were mounted on gelatinized slides, and then dewaxed and rehydrated. Antigen retrieval for anti-CD4 antibodies (VMRD, clone DH29A, 1: 100), anti-CD8 (ABD Serotec, clone YTS169.4, 1:40), FOXP3 (Spring, clone SP97, 1: 500) was made with sodium citrate solution (pH = 6.0) in water bath (90°C/20 minutes). Blockages of endogenous peroxidase and nonspecific proteins were performed with commercial solutions from Novolink system (Leica Biosystems Newcastle Ltd, UK) according to the manufacturer's recommendations. Primary antibodies were incubated for 16 hours at 4°C and, thereafter, the secondary antibody and polymer (Novolink) were applied according to the manufacturer's recommendations. Counterstaining was done with Harris Hematoxylin and immunoreactivity was revealed with chromogenic solution of 3,3 'diaminobenzidine (DAB) from Novolink system for 3 minutes.

Histochemical staining of toluidine blue (1%) for identifying mast cells, congo red (0.5%) for identifying eosinophils and Sirius red to evidence areas of tumor fibrosis

were performed according to the methods already recommended by Prophet et al. (1992) and Meyerholz et al. (2010).

Images from the four histologic types of tumors were captured on a Spot Insight Color digital camera adapted to an Olympus BX- 40, through the capture software SPOT® version 3.4.5, to count the inflammatory cells and determine tumor fibrosis percentage. To evaluate the inflammatory infiltrate by H&E staining, images of eight hotspots from each slide were captured in 100x objective, followed by differential counting of lymphocytes, plasma cells, neutrophils and macrophages through ImageJ software version 1.41. The values obtained in the eight hotspots were summed to provide total value of each cell type and the medians of these values were used to classify the infiltrates as to their density (low, moderate and high) according to what was proposed by Estrela-Lima et al. (2010).

For counting of eosinophils, mast cells, $TCD8^+$, $TCD4^+$ and $FOXP3^+$ lymphocytes, images of five hotspots from each sample were captured in 40x objective, followed by counting of labeled cells through ImageJ software (version 1.41). The values obtained in the five hotspots were summed to obtain the total value of each cell type. Relationships between cell types ($CD8^+/FOXP3^+$, $CD4^+/CD8^+$, $CD4^+/FOXP3^+$, lymphocytes/plasma cells, etc.) were obtained for each sample for later univariate statistical analysis.

Regarding tumor fibrosis percentage, three images of each histologic type were captured in 10x objective and analyzed with ImageJ software (version 1.41). Tumor fibrosis percentage of each image was obtained and, from these three values, a mean was calculated for each sample.

Kruskal-Wallis and Mann-Whitney + KAPLAN -MEYER statistical tests were performed with GraphPad Prism software (version 6) to evaluate the relationship between cell types and different histologic types of mammary tumors. Evaluation of the relationship between tumor fibrosis and histologic types of mammary tumors was performed through parametric analysis of variance (ANOVA) and t-test. Multivariate evaluation encompassing all observed parameters was done through software R (version 3.2.4)

3. Results

The predominant cell type in the inflammatory infiltrate of all histologic types of mammary neoplasms was the lymphocyte, while the density of the other cell types varied according to the histological type.

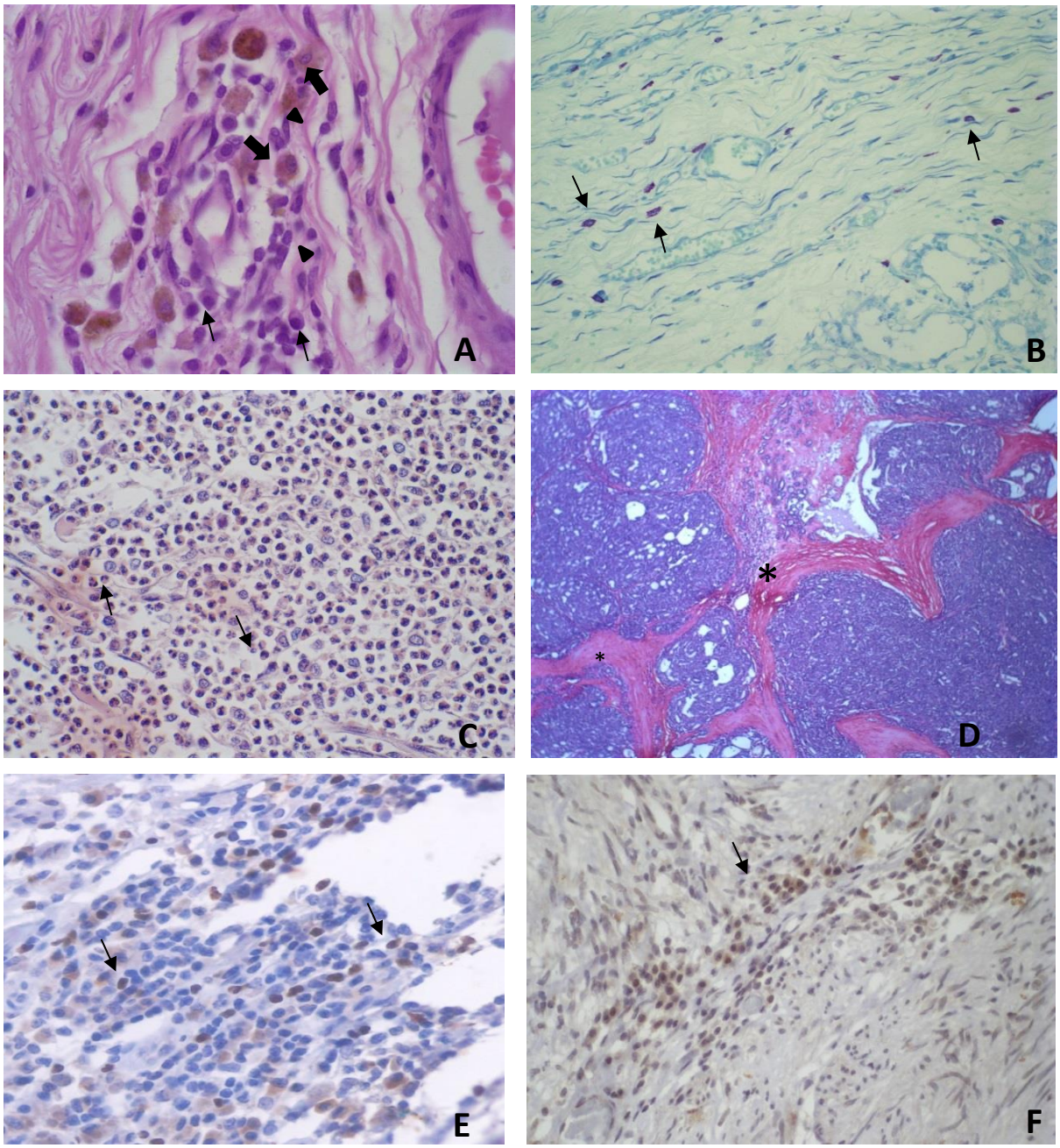

Figure 1. Photomicrographs showing inflammatory cells and tumor fibrosis through histochemistry and immunohistochemistry techniques. 1A: Mononuclear inflammatory infiltrate in peritumoral region. Fine arrow: Plasma cells. Broad arrow: Macrophages with hemosiderin. Arrowhead: lymphocytes (H&E; 100x). 1B: Presence of mast cells in peritumoral region (arrow) (toluidine blue 40x). 1C: Identification of infiltrated eosinophils in the tumor stroma (arrow) (red congo; 40x). D: Areas of intratumoral fibrosis (*) (red sirius; 10x). E: Arrow: positive cells for FOXP3 marker (counterstaining with Harris haematoxylin; 40x). F: Arrow: positive cells for CD4 marker observed in the tumor stroma (counterstaining with Harris Hematoxylin; 40x)

Comparison between different cell types and the four histological types showed that only plasma cells, neutrophils and macrophages presented statistically significant differences ($p < 0.05$) between two or more histological types (table 2).

Table 1 - Density of inflammatory cells in the four histological types of canine mammary neoplasms. Data presented in median (min. and max. values)

Histologic Tyipe/Cell Type	BMT	CMT	SC	TC	P value
Lymphocytes	51.00 (16.00;233.00)	97.50 (14,00;387.00)	141.00 (63.00;332.00)	143.00 (58.00;284.00)	0.22
Plasma cells	2.00 (0.0; 39.00)	22.00 (0.00;47.00)	33.50 (2.00;187.00)	17.00 (1.00; 41.00)	0.01
Neutrophils	0.00 (0.00; 0.00)	1.51 (0.00; 242.00)	0.00 (0.00; 1.00)	3.00 (0.00; 172.0,0)	0.00
Eosinophils	4.00 (0.00; 13.00)	5.50 (0.00; 84.00)	8.00 (0.00; 17.00)	13.00 (0.00; 185.00)	0.48
Mast cells	39.00 (4.00;75.00)	32.00 (10.00;77.00)	39.00 (4.00;75.00)	32.50 (10.00;77.00)	0.46
Macrophages	16.00 (0.00; 111.00)	25.00 (1.00;356.00)	1.00 (0.00; 11.00)	0.00 (0.00; 40.00)	0.05
CD4	42.00 (14.00;65.00)	53.00 (28.00;92.00)	67.00 (17.00;211.00)	67.00 (20.00;82.00)	0.34
CD8	43.00 (29.00;80.00)	67.00 (12.00;94.00)	81.50 (11.00;248.00)	35,00 (14.00;120.00)	0.41
FOXP3	22.00 (10.00;53.00)	31.50 (10.00;71.00)	29.00 (16.00;146.00)	24.00 (3,00;48.00)	0.57

BMT: benign mixed tumor; CMT: carcinoma in mixed tumor; SC: solid carcinoma; tubular carcinoma. Kruskal-Wallis nonparametric test

In the comparison of plasma cells medians between pairs of histological types, a significant difference (table 2) was observed for BMT x CMT and BMT x SC.

Table 2 – P values and median values (min. and max.) obtained from the inflammatory infiltrate profile and from tumor fibrosis percentage when pairs of groups (histologic types) were compared

Cell type	BMT x CMT	BMT x SC	BMT xTC	CMT x SC	CMT x TC	SC x TC
Plasma cells	0.00	0.01	0.01	0.14	0.59	0.13
	2.00; 22.00	2.00; 33.50	2.00; 17.00	22.00; 33.50	22.00; 17.00	33.50; 17.00
Neutrophils	0.00	≥0.99	0.07	0.00	0.82	0.04
	0.00; 1.50	0.00; 0.00	0.00; 3.00	1.50; 0.00	1.50; 3.00	0.00; 3.00
Macrophages	0.35	0.17	0.23	0.00	0.00	0.84
	16.00; 25.00	16.00; 1.00	16.00; 0.00	25.00; 1.00	25.00; 0.00	0.10; 0.00
Fibrosis (P)	0.05	0.27	0.01	0.23	0.6	0.06
Mean ± standard-deviation	9.63 ± 4.60	3.39 ± 2.88	12.17 ± 3.94	-6.24 ± 4.97	2.54 ± 4.69	8.78 ± 4.16

BMT: benign mixed tumor; CMT: carcinoma in mixed tumor; SC: solid carcinoma; TC: tubular carcinoma. Mann-Whitney nonparametric test for cell profile and T Test for fibrosis.

Solid carcinomas and carcinomas in mixed tumors presented the highest numbers of plasma cells associated with tumor inflammatory infiltrate (Table 1). Albeit, in the comparison of plasma cells numbers between pairs of groups, a statistical significance was found for BMT x CMT and BMT x SC as observed in table 2.

The relationship between number of neutrophils and histologic types presented statistical significance in the comparison between TMB x CTM, CTM x CS and CS x T (table 2)

Macrophages were found in less aggressive histologic types, such as BMT and CMT (table 1). Regarding the relationship between the number of macrophages and pairs of histologic types of tumors, it was possible to notice a statistical significance (table 2) in CMT x SC and CMT x TC.

In the evaluation of tumor fibrosis percentage for all histologic types, it was observed that tubular carcinomas presented the highest values. When fibrosis was compared between different tumors, significant differences (table 2) were found between BMT x CMT and BMT x TC.

The ratios between the inflammatory cell types that presented statistical significance in regard with the histologic types were lymphocytes/neutrophils, plasma cells/neutrophils, macrophages/neutrophils, macrophages/mast cells, as it is seen in table 3.

Table 3 - Ratio between cell types (median) for each histologic type and p values found through Kruskal-Wallis nonparametric test.

Ratio	BMT	CMT	SC	TC	P
Lymph/Plasm	6.75	4.06	4.24	6.50	0.512
	(0.00; 51.00)	(0.00; 37.75)	(1.03; 38.00)	(3.41; 143.00)	
Lymph/Mac	1.27	5.68	14.30	0.00	0.331
	(0.00; 116.50)	(0.04; 25.50)	(0.00; 130.00)	(0.00; 54.25)	
Lymph/Neut	0.00	53.50	0.00	0.01	0.002
	(0.00; 0.00)	(0.00; 178.50)	(0.00; 130.00)	(0.00; 2.96)	
Lymph/Eosi	7.40	15.20	13.83	8.20	0.574
	(0.00; 25.50)	(0.00; 89.25)	(0.00; 41.50)	(0.00; 56.80)	
Lymph/Mast	1.60	2.87	2.53	4.93	0.553
	(0.41; 12.75)	(0.32; 21.50)	(1.20; 10.85)	(0.92; 8.18)	
Plasm/Eosi	0.50	3.52	3.30	1.30	0.032
	(0,00; 2,00)	(0,00; 34,00)	(0,00; 14,10)	(0,00; 10,25)	
Plasm/Mast	0,16	0,78	0,56	0,50	0,072
	(0,00; 1,69)	(0,00; 1,67)	(0,28; 2,71)	(0,03; 1,51)	
Plasma/Neut	0.00	8.08	0.00	0.09	0.002
	(0.00; 0.00)	(0.00; 47.00)	(0.00; 0.00)	(0.00; 4.14)	
Mac/Plasm	0,05	0,86	0,02	0,00	0.010
	(0.00; 55.50)	(0.00; 17.80)	(0.00; 0.19)	(0.00; 2.85)	
Mac/Neut	0.00	7.13	0.00	0.00	0.001
	(0.00; 0.00)	(0.00; 356.00)	(0.00; 22.00)	(0.00; 1.05)	
Mac/Mast	0.59	0.81	0.01	0.00	0.003
	(0.00; 2,84)	(0.03; 10.20)	(0.00; 0.52)	(0.00; 1.42)	
Mac/Eosi	0.00	1,90	0,06	0,00	0.003
	(0.00; 11.25)	(0.00; 118.70)	(0.00; 0.50)	(0.00; 1.00)	
Mast/Eosi	3.00	3.75	5.14	3.2	0.782
	(0.00; 12.50)	(0.00; 44.00)	(0.00; 17.37)	(0.00; 16.20)	
CD4/CD8	0.58	0.84	0.77	1.25	0.225
	(0.32; 1.75)	(0.47; 7.67)	(0.45; 3.72)	(0.64; 4.00)	
CD4/FOXP3	1.33	2.01	1.56	2.79	0.112
	(0.87; 6.20)	(0.80; 6.40)	(0.77; 3.69)	(1.60; 1.66)	
CD8/FOXP3	2.58	2.45	2.39	2.50	0.981
	(0,83; 3,70)	(0,17; 8,60)	(0,29; 5,89)	(0,75; 5,33)	

BMT: benign mixed tumor; CTM: carcinoma in mixed tumor; SC: solid carcinoma; TC: tubular carcinoma.

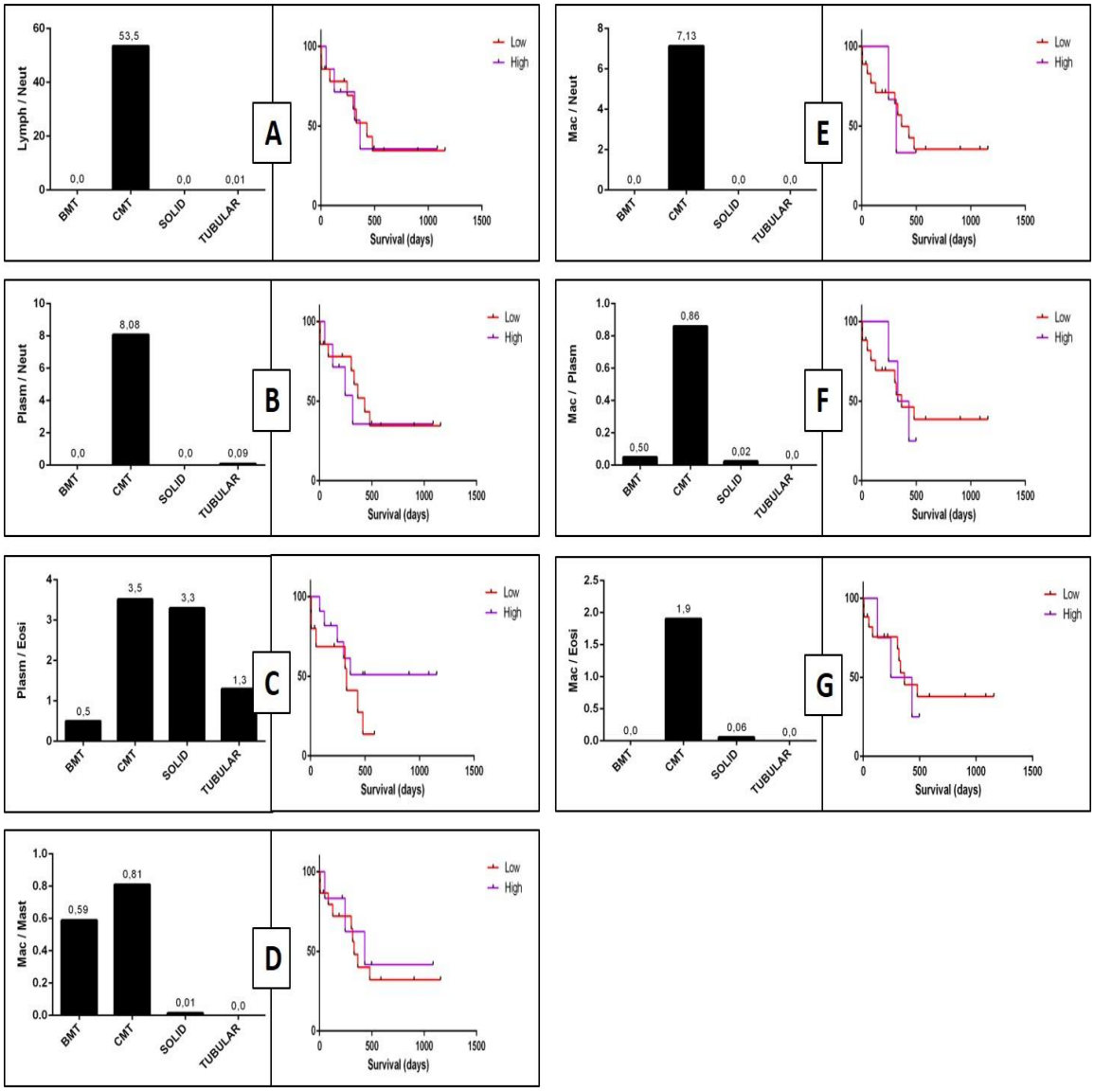

Figure 6 - Significant ratios between cell types (median values) with their respective survival curves. Kruskal-Wallis nonparametric test. 6A: Lymphocytes/neutrophils ratio. 6B: Plasma cells / neutrophils ratio. 6C: Plasma cells / eosinophils ratio. 6D: Macrophages / mast cells ratio. 6E: macrophages / plasma cells ratio. 6F: macrophages/eosinophils ratio. 6G: Macrophages / neutrophils ratio.

Furthermore, when evaluating the previous significant ratios between pairs of histologic types, we noticed that tubular carcinomas were the ones that showed statistical significance in almost every analysis, as it is shown in table 4.

Table 4: Comparison of statistically significant ratios between histologic types of canine mammary neoplasms. Nonparametric Mann-Whitney test.

Ratio	BMT x CMT	BMT x SC	BMT x TC	CMT x CS	CMT x TC	SC x TC
Lymph/Neut	0.00	0.47	0.07	0.07	0.00	0.57
	53.50	0.00	0.01	-13.25	-52.02	0.00
Plasm/Eosi	0.00	0.03	0.55	>0.09	0.08	0.13
	2.71	2,82	0,07	0,0	-2,51	-2,38
Plasm/Neut	0.00	>0.99	0.07	0.02	0.05	0.18
	8.08	0.00	0.09	-7.66	-7.43	0.04
Mac/Eosi	0.25	0.65	0.34	0.00	0.00	0.65
	0.82	0.00	0.00	-1.75	-1.81	0.00
Mac/Neut	0.00	0.47	0.46	0.02	0.01	>0.99
	7.13	0.00	0.00	-6.63	-6.66	0.00
Mac/Plasm	0.54	0.54	0.416	0.00	0.01	0.79
	0.13	-0.02	-0.05	-0.76	-0.64	0.00
Mac/Mast	0.271	0.135	0.291	0.000	0.005	>0.999
	0.40	-0.55	-0.45	-0.62	-0.61	0.00

BMT: benign mixed tumor; CMT: carcinoma in mixed tumor; SC: solid carcinoma; TC: tubular carcinoma; Lymph: Lymphocyte; Neut: Neutrophil; Plasm: Plasma cell; Eosi: Eosinophil; Mac: Macrophage; Mast: Mast Cell.

The multivariate statistical analysis involving the same aforementioned parameters showed three correspondences. The first correspondence involved benign mixed tumors, low lymphocyte density, low plasma cell density and low tumor fibrosis percentage. The second correspondence involved tubular carcinomas, low $CD8^+$ cell density and high neutrophil density. The third one showed a relationship between absence of invasion or micro invasion areas, low density of $CD4^+$ cells and moderate density of $CD8^+$ cells.

4.Discussion

Among the inflammatory cells identified in the present study, lymphocytes were the most numerous in all histologic types of canine mammary neoplasms. Although Saeki et al. (2012) and Kim et al. (2013) found a relationship between high density of lymphocytes in the inflammatory infiltrate and aggressiveness in canine mammary tumors, the present study did not verify such relationship, but complemented these data by showing, through multivariate statistical analysis, a relation between low density of lymphocytes and less aggressive canine mammary tumor, such as benign mixed tumors. Furthermore, cytotoxic T and T helper subtypes were also related to some parameters as discussed below.

According to the studies of Mahmoud et al. (2011), Miyashita et al. (2015) and Preston et al. (2013), the increase in cytotoxic T lymphocytes ($CD8^+$) is associated with better prognosis, especially in the $CD8^+/FOXP3^+$ ratio. In the present study, multivariate statistical analysis showed an important association between tubular carcinomas and low $CD8^+$ cells. This finding reflects the importance of cytotoxic T lymphocytes in the fight against tumor cells and reinforces the immunomodulatory action of aggressive neoplasias, which may be directly responsible for the low number of $CD8^+$ cells in the inflammatory infiltrate.

However, the high density of T helper lymphocytes ($CD4^+$) was associated to both presence of metastases and lower survival rates, especially in the presence of low density of cytotoxic T lymphocytes ($CD8^+$), according to Macchetti et al. (2006), Estrela-Lima et al. (2010) and Carvalho et al. (2014). In the present study, no relationship was found between $CD4^+/CD8^+$ ratio and the parameters evaluated above. However, it was verified that a low density of $CD4^+$ cells in canine mammary neoplasias was related to the absence of invasion or microinvasion areas, leading to the conclusion that $CD4^+$ cells may play a role, even indirectly, in metastatic mechanisms.

Regarding the macrophages, a higher number of these cells were found in malignant tumors with better prognosis (CMT), but no relationship with survival, invasion and metastasis was observed. These findings contradict what was pointed out by Król et al. (2011, 2014) and Raposo et al. (2014), who verified increase of tumor associated macrophages (TAMs) related to increased invasion and metastasis and decreased survival in patients, which are characteristics of more aggressive neoplasias. However, it is important to point out that, in the present study, immunohistochemistry technique was not used to identify and differentiate

subpopulations of M1 or M2 macrophages. The fact that M1 macrophages are related to innate immunity and, therefore, aimed at cytotoxic action, may justify a greater presence of this immunophenotype in carcinomas in mixed tumors, showing a relationship between the high intensity of macrophages and less aggressive tumors. An immunohistochemical characterization of macrophages in carcinomas in mixed tumors would be interesting to verify this hypothesis.

Higher numbers of neutrophils appeared mainly in carcinomas in mixed tumors and tubular carcinomas. In multivariate analysis, high neutrophil density showed relation to tubular carcinomas. This data suggests a relation with more aggressive neoplasias, as described by Nozawa et al. (2006), Youn and Gabrilovich (2010). However, solid carcinomas were also expected to exhibit large neutrophil populations, since it was the most aggressive histologic type of this study. Instead, carcinomas in mixed tumors were the neoplasms that showed density of neutrophils similar to tubular carcinomas. Nevertheless, significant results for lymphocyte/neutrophil and plasma cell/neutrophil ratios in carcinomas in mixed tumors indicate that higher proportions of lymphocytes and plasma cells over neutrophils are related to less aggressive neoplasms, suggesting that these three cell types are closely related in the development and progression of neoplasms.

The values that were found for plasma cells do not agree with the data of Yoon et al. (2011), who found an association between presence of plasma cells and better prognosis in human breast cance and Knief et al. (2016), that verified prolonged survival in human patients with high density of plasma cells and B lymphocytes in esophagogastric transition adenocarcinoma. However, it is important to point out that, in the comparison between plasma cells and eosinophils densities, carcinomas in mixed tumors and solid carcinomas were the histologic types that presented the highest ratios. When evaluated as an isolated parameter, plasma cells have been shown to be associated with more aggressive neoplasms. Besides, it is important to point out that the number of plasma cells associated with low number of eosinophils may imply even more aggressive neoplasms.

As in the studies of De Kruijf et al. (2011) and Gujam et al. (2014), which showed a relationship between higher area of tumor fibrosis and human patients with worse prognosis, the present study suggests that this relationship may also be true for more aggressive canine mammary neoplasias, such as tubular carcinomas. In addition, multiple correspondence analysis showed a relationship between low fibrosis

percentage and benign mixed tumors, reinforcing the importance of fibrosis in tumor progression.

Another important observation is that, although solid carcinomas are the most aggressive tumors among the histologic groups, the tumor fibrosis percentage in this group was lower than in tubular carcinomas and even in carcinomas in mixed tumors. This can be explained by the characterization of solid carcinomas, which consists of proliferation of organized epithelial cells in a solid array with a quantity of stroma varying from little to moderate (CASSALI, 2002). Another important factor that concerns the difference observed in carcinomas in mixed tumors is the histological characterization of these neoplasias, which includes the benign proliferation of mesenchymal cells with formation of cartilage or bone, possibly combined with the formation of myxoid fibrous tissue (MISDORP, 1999; CASSALI et al., 2002), justifying a larger area of tumor fibrosis.

5.Conclusion

The results presented in this study suggest that high densities of neutrophils and plasm cells are related to more aggressive mammary neoplasms, while high densities of macrophages and $TCD8^+$ cells and low density of $TCD4^+$ lymphocytes are associated with less aggressive neoplasms. The tumor fibrosis percentage has been shown to be a characteristic related to more aggressive mammary neoplasias, such as tubular carcinomas, which may indicate its use for prognostic purposes also in veterinary medicine.

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