University of Trás-os-Montes and Alto Douro

Advances in Canine Mammary Cancer: A Role for Inflammatory Infiltrate in Tumor Microenvironment

PhD Thesis in Veterinary Sciences

Maria Isabel da Silva Carvalho

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Vila Real, 2017

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Vila Real, 2017

I declare for all due purposes that the PhD thesis meets the technical and scientific standards required by the regulations of the University of Trás-os-Montes and Alto Douro. The presented doctrines are the exclusive responsibility of the author.

This thesis was specifically prepared to obtain the PhD degree in Veterinary Sciences.

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"Always do your best. What you plant now, you will harvest later."

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To my parents for the dedication,

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Abstract

Cancer related inflammation is part of the major cancer hallmarks and has an important role in mammary carcinogenesis being involved in tumor aggressiveness and poor clinical outcome. In dogs only few studies have yet concentrated on the influence of immune cells in clinical outcome of dog mammary tumor patients. In this context, the present work was conducted with the main aim of up-to-date knowledge about the roles of the intertwined signaling pathways shared by T-lymphocytic/macrophage infiltrates and important tissue biomarkers in canine mammary tumors (CMT) progression, aggression and prognosis.

Immunosuppression associated with tumor infiltrating T-lymphocytes (TILs) has been explained by the compartment of regulatory T-cells (Treg) that inhibit anti-tumor cytotoxic activities. Based on these evidences, first, we assessed, by immunohistochemistry, the characterization of intratumoral Treg cells, as well as some cytokines related by them: TGFβ and IL-35. Our results demonstrated that FoxP3 was present in tumors with more aggressive phenotypes: high histological grade of malignancy (HGM), presence of neoplastic intravascular emboli and presence of lymph node metastasis. Additionally, also showed that intratumoral $FoxP3^+$ Treg cells were associated with shorter overall survival (OS), both in univariate and multivariate analysis. Tregs can secrete inhibitory cytokines (TGFβ and IL-35), inducing tumors tolerance. Present work suggests a link between TGFβ and parameters of tumor aggressiveness, reflecting its involvement in CMT malignant transformation. Our data demonstrated also a positive correlation between intratumoral FoxP3, TGFβ levels, VEGF and CD31. Moreover tumors with abundant TGFβ and with concurrent high expression of TGFβ/FoxP3, FoxP3/VEGF, TGFβ/VEGF were associated with shorter OS time and the TGFβ/FoxP3 tumors class retained the association with worse survival in multivariate analysis, arising as an independent predictor of poor prognosis. IL-35 is a Treg cell-secreted cytokine that inhibits T-cells proliferation and function and to the best of our knowledge, this is the first study that investigates the role of IL-35 in CMT development and clinical outcome. Our results showed that IL-35 overexpression was significantly associated with advancement of tumor stage and unfavorable prognosis by univariate and multivariate survival analysis. Interestingly our findings indicate, for the first time in dog mammary tumors, the independent prognostic value of $FoxP3^+$ Treg cells and

TGFβ/FoxP3 tumors class. Additionally the IL-35 seems to be a new biomarker to predict the CMT clinical outcome.

For a more comprehensive approach about the role of immune cells in dog mammary carcinogenesis, we perform a wide study focused on the intertwined signaling pathways shared by T-lymphocytic/macrophage infiltrates and important tissue biomarkers in mammary tumor microenvironment. We focused on relevant cell biomarkers in mammary carcinogenesis, the COX-2, EGFR and c-kit which are often overexpressed or mutated in this type of tumor. Present data demonstrated a significant association of high COX-2 immunoexpression with $CD3^+$ T-lymphocytes and MAC387 macrophages. Tumors with concurrent high COX-2/CD3 and high COX-2/MAC expression were associated with variables of tumor aggressiveness and shorter OS. Current results also demonstrated that the concurrent $COX\text{-}2^+/EGFR^+$ expression was associated with higher numbers of intratumoral $CD3^+$ T-cells. Furthermore a significant association and a positive correlation between $CD3^+$ T-lymphocytes and c-kit expression were observed. Tumors with high c-kit expression showed higher counts of $CD3^+$ T-cells and were associated with high HGM, presence of neoplastic intravascular emboli, presence of lymph node metastasis, angiogenesis and shorter OS. The findings indicate that COX-2, EGFR and c-kit pathways are important not only for the remodeling of mammary tumor microenvironment but also could be a very important targets for tumor immunological therapy.

Finally, in an in vitro co-culturing experiment, was our aim to prove the potential bidirectional crosstalk between tumor cells and immune cells on COX-2 regulation. Our data showed that co-culturing of canine mammary carcinoma cell line Sh1b and canine peripheral blood mononuclear cells (PBMCs) induced a trend of COX-2 overexpression in mammary cancer cells. In turn, COX-2 expression by PBMCs, among them predominantly $CD68^+$ macrophages, was significantly attenuated by co-culture with Sh1b. In accordance, co-culture with $CD68^+$ differentiated THP1 (dTHP1) prompted an intracellular production of COX-2 in Sh1b cells. The intracellular COX-2 expression from dTHP1 decreased when they were treated with conditioned medium from cultured Sh1b cells. Present results represents a significant advance on understanding the possible role of COX-2 in inducing a cancer tolerogenic microenvironment in CMT namely through a cancer associated macrophages immunomodulation.

Overall present work demonstrated that, similarly to human breast cancer, also in CMT the inflammatory responses in mammary cancer sites are able to orchestrate hallmark-facilitating programs in tumor microenvironment.

Keywords: Canine mammary tumors; Macrophages; Prognosis; T-lymphocytes; Treg cells; Tumor microenvironment.

Resumo

A inflamação faz parte dos principais "hallmarks" associados ao cancro e tem um papel relevante na carcinogénese mamária, relacionando-se com a agressividade tumoral e pior prognóstico. Nos tumores mamários dos canídeos (TMC) os estudos que se debruçam sobre o efeito das células imunitárias no prognóstico são escassos. Neste contexto, o presente trabalho foi realizado com o objetivo de fomentar o conhecimento sobre o papel das vias de sinalização compartilhadas pelo infiltrado de linfócitos T/macrófagos e importantes biomarcadores tumorais na progressão, agressividade e prognóstico destes tumores.

A imunossupressão associada ao infiltrado de linfócitos T no tumor tem sido explicada pela capacidade inibitória das células T reguladoras (Treg) que impedem as atividades citotóxicas anti-tumorais. Com base nestas evidências, caracterizámos por imunohistoquímica as células Treg intratumorais ($FoxP3^+$), bem como algumas citoquinas relacionadas com elas: TGFβ e IL-35. Os nossos resultados demonstraram que a maior imunorreactividade para o FoxP3 está presente em tumores com fenótipos mais agressivos: elevado grau histológico de malignidade (GHM), presença de embolos intravasculares e metástases nos linfonodos. Adicionalmente, mostrámos que as células Treg $FoxP3^+$ intratumorais estão associadas com uma menor sobrevida total (ST), por análise univariada e multivariada. As células Treg secretam citoquinas inibidoras (TGFβ e IL-35), induzindo a tolerância aos tumores. Os nossos resultados sugerem uma associação entre o TGFβ e parâmetros de agressividade tumoral, refletindo o seu envolvimento na transformação maligna. Demonstraram também uma correlação positiva entre o FoxP3 intratumoral, os níveis de TGFβ, VEGF e CD31. Além disso, tumores com abundante TGFβ e com elevada expressão simultânea de TGFβ/FoxP3, FoxP3/VEGF, TGFβ/VEGF foram associados ao menor tempo de ST e a classe de tumores TGFβ/FoxP3 elevados revelou-se como um fator independente de mau prognóstico na análise multivariada. A IL-35 é uma citoquina secretada pelas células Treg que inibe a proliferação e função das células T. Demonstrámos que a sobre-expressão da IL-35 está associada com o estádio clínico mais avançado e com um prognóstico desfavorável na análise univariada e multivariada. Foi possível constatar, pela 1ª vez nos TMC, o valor prognóstico independente das células $FoxP3^+$ Treg e da classe de tumores TGFβ/FoxP3

elevados. A IL-35 evidenciou-se ainda como um novo biomarcador capaz de prever a evolução clínica dos TMC.

No seguimento deste trabalho, realizámos um amplo estudo que incidiu sobre as vias de sinalização compartilhadas pelo infiltrado de linfócitos T/macrófagos e importantes biomarcadores tumorais no microambiente dos TMC. Com esse propósito estudámos biomarcadores celulares importantes na carcinogénese mamária (COX-2, EGFR e c-kit), que estão frequentemente sobre-expressos ou mutados nos TMC. Constatámos uma associação significativa da imunoexpressão elevada da COX-2 com os linfócitos T $CD3^+$ e macrófagos MAC387. Tumores com elevada expressão de COX-2/CD3 e COX-2/MAC estão associados com variáveis de agressividade tumoral e menor ST. Demonstrámos ainda que a expressão simultânea de $COX\text{-}2^+/EGFR^+$ está associada com maior número de células T $CD3^+$ intratumorais e foi observada uma associação significativa e uma correlação positiva entre os linfócitos T $CD3^+$ e a expressão de c-kit. Tumores com alta expressão de c-kit apresentaram maior número de células T $CD3^+$ estando associados a um GHM elevado, presença de êmbolos intravasculares, metástases nos linfonodos e menor ST. Os resultados demonstram que as vias de sinalização que envolvem a COX-2, o EGFR e o c-kit são importantes, não só para a remodelação do microambiente do tumor mamário, mas também como alvos para a terapia imunológica anti-tumoral.

Para complementar o trabalho, foi realizada uma experiência de co-cultura *in vitro* onde se pretendia demonstrar a potencial participação bidirecional das células tumorais e células imunitárias na regulação da COX-2. Os resultados mostraram que a co-cultura entre a linha celular de carcinoma mamário de cão Sh1b e as células mononucleares de sangue periférico de cão (PBMCs) induziram uma tendência para a sobre-expressão de COX-2 nas células cancerígenas. Por sua vez, a expressão de COX-2 pelos PBMCs, entre eles predominantemente macrófagos $CD68^+$, foi significativamente atenuada pela co-cultura com Sh1b. Em conformidade, a co-cultura com $CD68^+$ THP1 diferenciada (dTHP1) provocou um aumento da produção intracelular de COX-2 pelas células Sh1b. A expressão intracelular de COX-2 pela dTHP1 diminuiu quando estas células foram tratadas com meio condicionado das células Sh1b. Estes resultados representam um avanço significativo na compreensão do papel da COX-2 na remodelação do microambiente tumoral nos TMC, nomeadamente através da imunomodulação dos macrófagos associados ao tumor.

Globalmente, o presente trabalho demonstrou que, à semelhança do cancro de mama da mulher, também nos TMC as células inflamatórias no microambiente do tumor são capazes de orquestrar programas facilitadores da progressão tumoral.

Palavras-chave: Tumores mamários dos canídeos; Macrófagos; Prognóstico; Linfócitos T; células Treg; Microambiente tumoral.

Thesis Outline

Cancer related inflammation is part of the major cancer hallmarks and has an important role in mammary carcinogenesis being involved in tumor aggressiveness and poor clinical outcome. In dogs only few studies have yet concentrated on the influence of immune cells in clinical outcome of dog mammary tumor patients. In this context, the present work was conducted with the main aim of up-to-date knowledge about the roles of the intertwined signaling pathways shared by T-lymphocytic/macrophage infiltrates and important tissue biomarkers in canine mammary tumors (CMT) progression, aggression and prognosis.

The present thesis was divided into five chapters. The main motivation that conducted this work was the need to better understanding the influence of immune cells in dog mammary carcinogenesis and to establish new prognostic factors and molecular therapeutic targets that could be of value in the treatment of CMT.

Chapter I is a general introduction on the state of the art about CMT pathophysiology. Additionally it was describes the intertwined signaling pathways shared by T-lymphocytic/macrophage infiltrates and important tissue biomarkers in both human and dog mammary carcinogenesis.

Chapter II encompass original data focusing on the characterization of intratumoral FoxP3 regulatory T-lymphocytes, TGFβ and IL-35 immunoexpression and attempt to clarify the role of these molecular markers in CMT aggressiveness and prognosis.

Chapter III includes original data assessing the relationship between T-lymphocytic/macrophage infiltrate and emergent molecular targets. There is a growing list of signaling molecules released by inflammatory cells that serve as effectors of their tumor-promoting actions. This chapter emphasizes the important association of intratumoral T-lymphocytes/macrophages with COX-2, EGFR and c-kit that seems to contribute to the amplification of the tumoral inflammatory state related with cancer progression and worse clinical outcome.

Chapter IV focuses on the determination, in an *in vitro* co-culturing study, of the bidirectional COX-2 regulation between cancer cells and monocytes/macrophages in CMT. This original data represent a significant advance on understanding the possible

role of COX-2 in inducing a cancer tolerogenic microenvironment namely through cancer associated macrophages immunomodulation.

A general discussion of the results and concluding remarks are presented in Chapter V.

Publications and Communications

The author of this thesis declares to have actively participated in the elaboration and execution of experimental work that led to the results presented, which originated several publications in international scientific journals with Referee, as well as oral and poster communications in national and international meetings.

Publications in international scientific journals with Referee

Maria Isabel Carvalho, Isabel Pires, Justina Prada, Felisbina Luísa Queiroga (2014). **A Role for T-Lymphocytes in Human Breast Cancer and in Canine Mammary Tumors**. *BioMed Research International*. doi: 10.1155/2014/130894

Maria Isabel Carvalho, Isabel Pires, Justina Prada, Adriano Fernandes Ferreira, Felisbina Luísa Queiroga (2015). **Positive Interplay between $CD3^+$ T-lymphocytes and Concurrent COX-2/EGFR expression in Canine Malignant Mammary Tumors**. *Anticancer Research.* 35(5): 2915-2920. doi: 10.4142/jvs.2015.16.2.225

Maria Isabel Carvalho, Isabel Pires, Marlene Dias, Justina Prada, Hugo Gregório, Luís Lobo, Felisbina Luísa Queiroga (2015). **Intratumoral $CD3^+$ T-lymphocytes immunoexpression and its association with c-kit, Angiogenesis, and Overall Survival in Malignant Canine Mammary Tumors**. *Analytical Cellular Pathology*. Article ID 920409. doi: 10.1155/2015/920409

Maria Isabel Carvalho, Isabel Pires, Justina Prada, Teresa Raposo, Hugo Gregório, Luís Lobo, Felisbina Luísa Queiroga (2016). **High COX-2 expression is associated with increased Angiogenesis, Proliferation and Tumoral Inflammatory Infiltrate in Canine Malignant Mammary Tumors: a Multivariate Survival Study**. *Veterinary and Comparative Oncology*. doi: 10.1111/vco.12206.

Maria Isabel Carvalho, Isabel Pires, Justina Prada; Hugo Gregório; Luís Lobo; Felisbina Luísa Queiroga (2016). **Intratumoral FoxP3 expression is associated with Angiogenesis and Prognosis in Malignant Canine Mammary Tumors**.

Veterinary Immunology and Immunopathology. 178: 1-9. doi: 10.1016/j.vetimm.2016.06.006

Maria Isabel Carvalho, Ricardo Silva-Carvalho, Isabel Pires, Justina Prada, Rodolfo Bianchini, Erika Jensen-Jarolim, Felisbina Luísa Queiroga (2016). **A Comparative Approach of Tumor Associated Inflammation in Mammary Cancer between Humans and Dogs**. *BioMed Research International. doi: 10.1155/2016/4917387*

Maria Isabel Carvalho, Isabel Pires, Justina Prada, Carla Pinto, Hugo Gregório, Luís Lobo, Felisbina Luísa Queiroga. **Crosstalk between TGFβ, FoxP3 expression and Angiogenesis in Malignant Canine Mammary Tumors: association with Clinicopathological Parameters and Prognosis**. Submitted manuscript

Maria Isabel Carvalho, Rodolfo Bianchini, Judit Fazekas, Ina Herrmann, Irene Flickinger, Johann G. Thalhammer, Isabel Pires, Erika Jensen-Jarolim, Felisbina Luísa Queiroga. **Bidirectional Regulation of COX-2 expression between Cancer Cells and Macrophages**. Submitted manuscript

Maria Isabel Carvalho, Isabel Pires, Justina Prada, Hugo Gregório, Carla Pinto, Felisbina Luísa Queiroga. **Assessing the Interleukin 35 immunoexpression in Malignant Canine Mammary Tumors: association with Clinicopathological Parameters and Prognosis**. Submitted manuscript

Book chapters

Maria Isabel Carvalho, Teresa Raposo, Helena Rodrigues, Isabel Pires, Justina Prada, Felisbina L. Queiroga (2014). **Neoplastic Diseases of the Canine Mammary Gland**. In Book: Mammary Glands: Anatomy, Development and Diseases, Edmund B. Rucker (Ed), Nova Science Publishers, Inc, NY. ISBN: 978-1-62948-856-1 eBook

Oral communications

Maria Isabel Carvalho, Isabel Pires, Justina Prada, Felisbina Luísa Queiroga. **The interplay between $CD3^+$ T-lymphocytes and Concurrent COX-2/EGFR**

expression in Canine Malignant Mammary Tumors. 9th International Conference of Anticancer Research, 6-10 October, 2014, Sithonia, Greece.

Poster communications

Maria Isabel Carvalho, Isabel Pires, Justina Prada, Teresa Raposo, Helena Rodrigues, Felisbina Luísa Queiroga. **High COX-2 expression is associated with increased Angiogenesis, Proliferation and Tumoral Inflammatory Infiltrate in Canine Mammary Tumors**. European Society of Veterinary Oncology Congress, 22-24 May, 2014, Vienna, Austria.

Maria Isabel Carvalho, Marlene Dias, Isabel Pires, Justina Prada, Felisbina Luísa Queiroga. **Intratumoral $CD3^+$ T-lymphocytes immunoexpression and its association with c-kit and Angiogenesis in Malignant Canine Mammary Tumors: a Multivariate Survival Analysis**. European College of Veterinary Internal Medicine-Companion Animals, ECVIM-CA Congress, 10-12 September, 2015, Lisbon, Portugal.

Maria Isabel Carvalho, Isabel Pires, Justina Prada, Felisbina Luísa Queiroga. **Intratumoral FoxP3 expression in Malignant Canine Mammary Tumors: its association with Clinicopathological Parameters, Angiogenesis and Prognosis**. European College of Veterinary Internal Medicine-Companion Animals, ECVIM-CA Congress, 10-12 September, 2015, Lisbon, Portugal.

Awarded works

ESVONC Best Poster Communication

Maria Isabel Carvalho, Isabel Pires, Justina Prada, Felisbina Luísa Queiroga. **Intratumoral FoxP3 expression in Malignant Canine Mammary Tumors: its association with Clinicopathological Parameters, Angiogenesis and Prognosis**. European College of Veterinary Internal Medicine-Companion Animals, ECVIM-CA Congress, 10-12 September, 2015, Lisbon, Portugal.

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List of Abbreviations

A

AKT or PKB, Protein kinase B

ANOVA, Analysis of variance

AP-1, Activator protein 1

B

BRCA1, Breast cancer susceptibility gene-1

BT474, Human breast carcinoma cell line

C

cAMP, Cyclic adenosine monophosphate

CCL2/MCP-1, Monocyte chemotactic protein -1

CCL8 or MCP-2, Monocyte chemotactic protein 2

CCL18, Chemokine (C-C motif) ligand 18

CCR, C-C chemokine receptor

CD31, Platelet Endothelial Cell Adhesion Molecule 1- PECAM 1

CD3, Cluster of differentiation 3

CD4, Cluster of differentiation 4

CD8, Cluster of differentiation 8

CK5, Cytokeratin 5

c-kit, Tyrosine-protein kinase Kit

CMT, Canine mammary tumors

COX-2, Cyclooxygenase 2

CSF1, Macrophage colony-stimulating factor 1

D

DCs, Dendritic cells

DFS, Disease-free survival

DMEM, Dulbecco's modified Eagle's medium

E

EGF, Epidermal growth factor

EGFR, Epidermal growth factor receptor (ErbB1 or HER-1)

EMT, Epithelial-to-mesenchymal transition

Ephs, Ephrin receptors

ER, Estrogen receptor

F

FCS, Fetal calf serum

FGF, Fibroblast growth factor

FoxP3, Forkhead box P3

G

G-CSF, Granulocyte colony-stimulating factor

GH, Growth hormone

H

HBSS, Hank's Balanced Salt Solution

HER-2/neu, Human epidermal growth factor receptor 2 (ErbB2)

HGF/SF, Hepatocyte growth factor/scatter factor receptor

HGM, Histological grade of malignancy

HIF-1α, Hypoxia-inducible factor 1α

HT29, Human colon cancer cell line

I

IFN-γ, Interferon gamma

IGF-1, Insulin-like growth factor 1

IL, Interleukin

M

MAPK/ERK1/2, Mitogen activated protein kinase

M-CSF, Macrophage colony-stimulating factor

MDSCs, Myeloid-derived suppressor cells

MEK p44/42, Mitogen-activated protein kinase

MHC, Major histocompatibility complex

MIF, Macrophage migration inhibitory factor

MMP, Matrix metalloproteinase

MVD, Microvessel density

N

n, Number of observations

NFκB, Nuclear factor kappa-B

NK, Natural killer

O

OS, Overall survival

OVH, Ovariohysterectomy

P

PBMCs, Peripheral blood mononuclear cells

PBS, Phosphate-Buffered Saline

PCNA, Proliferating cell nuclear antigen

PDGF, Platelet-derived growth factor

PGE2, Prostaglandin E2

PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase

PMA, Phorbol myristate acetate

PRL, Prolactin

p53, Tumor suppressor p53

p63, Transformation-related protein 63

R

Ras, Protein superfamily of small GTPases

RPMI, Roswell Park Memorial Institute medium

RTKs, Receptor tyrosine kinases

S

SCF, Stem cell factor

SD, Standard deviation

SE, Standard error

Sh1b, Canine mammary carcinoma cell line

STAT3, Signal transducer and activator of transcription 3

T

TAMs, Tumor associated macrophages

TCR, T-cell receptor

TGFβ, Transforming growth factor beta

TGFβR, Transforming growth factor beta receptor

Th1, T-helper 1 lymphocytes

Th2, T-helper 2 lymphocytes Th17, T-helper 17 lymphocytes

THP1, Human monocytic cell line

TILs, Tumor-infiltrating T-lymphocytes

TKIs, Tyrosine kinase inhibitors

TNFα, Tumor necrosis factor alpha

Tregs, Regulatory T-lymphocytes

U

uPA, Urokinase-type plasminogen activator

V

VEGF, Vascular endothelial growth factor VEGFR, Vascular endothelial growth factor receptor

W

WHO, World Health Organization

Others

5-LOX, 5-lipoxygenase

Chapter I - General Introduction

Book Chapter:

Neoplastic Diseases of the Canine Mammary Gland.

In Book: Mammary Glands: Anatomy, Development and Diseases, Edmund B. Rucker.

Maria Isabel Carvalho, Teresa Raposo, Helena Rodrigues, Isabel Pires, Justina Prada, Felisbina Luísa Queiroga.

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Review Articles:

A Role for T-lymphocytes in Human Breast Cancer and in Canine Mammary Tumors.

Maria Isabel Carvalho, Isabel Pires, Justina Prada, Felisbina Luísa Queiroga.

BioMed Research International, 2014

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A comparative approach of Tumor Associated Inflammation in Mammary Cancer between Humans and Dogs.

Maria Isabel Carvalho, Ricardo Silva-Carvalho, Isabel Piresa, Justina Prada, Rodolfo Bianchini, Erika Jensen-Jarolim, Felisbina Luísa Queiroga.

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I.1 Neoplastic Diseases of the Canine Mammary Gland

The development of the mammary gland is a puzzling phenomenon and the research on this field has been focused mostly on the carcinogenesis, with a less goal-oriented concern in basic histology. Canine mammary tumors (CMT) represent a serious problem in veterinary medicine [1]. Clinically, these tumors share many similar features with human breast cancer in terms of histopathology, biological behavior, hormone dependence, risk factors and genetic alterations. These similarities have led to a proposed canine model for the study of breast cancer in women [2-5].

Mammary Gland Development

The mammary gland is a modified apocrine sweat gland present in the subcutaneous connective tissue, containing a nipple and consisting of a network of ducts surrounded by a fibrovascular and adipocyte-rich stroma. Mammary development can first be recognized during the embryonic stages by the appearance of 2 ventral ridges of ectoderm, which cover the mesodermic regions from the axillary to the inguinal area. The ectodermal cells migrate along each milk line and coalesce in a complex interaction with the mesenchymal cells to form a placode, which eventually becomes an individual mammary gland [6]. At birth, the mammary gland is not fully developed. Its development continues at puberty, and throughout reproductive life the gland undergoes a morphogenetic process controlled by its hormonal environment. This involves complex interactions between ovarian steroid and polypeptide hormones and growth factors produced in successive estrous cycles. The last stages of development of the mammary gland occur in the adult female only during pregnancy, with the proliferation of the ductal tissue, differentiation into milk-producing acini, secretion of milk by the acinar cells, and, at the end of lactation, involution of the secretory component of the gland with preservation of the ductal structures [6, 7].

Mammary Gland Anatomy and Blood Supply

Most dogs develop 5 pairs of mammary glands, although 4 or 6 pairs have been found in a few animals. There are 2 thoracic (M1 and M2), 2 abdominal (M3 and M4), and 1 inguinal (M5) pair of mammary glands [8-10]. The blood supply in canine mammary glands derives from the sternal branches of the internal and lateral thoracic artery, intercostal, the cranial and caudal superficial epigastric arteries, deep cranial epigastric,

abdominal segmented and deep iliac circumflex. Venous drainage of the glands is similar to the arterial supply, though small veins may cross the midline between the right and left mammary glands [8, 11].

Lymph Drainage of the Mammary Gland

By convention, most malignant epithelial neoplasms (carcinomas) metastasize via the lymphatic system, whereas malignant mesenchymal neoplasms (sarcomas) metastasize via capillaries and veins. Patsikas and colleagues have methodically examined the lymph drainage of CMT located in each of the mammary glands [12] (**Table 1**).The lymph drainage of the fourth and fifth neoplastic mammary glands is directed to the ipsilateral superficial inguinal lymph nodes as sentinel lymph nodes of the fourth and fifth healthy mammary glands, whereas the two most cranial neoplastic mammary glands generally drain to the axillary lymph nodes. These authors also strength the clinician's notion that mammary carcinomas occurring in the inguinal (M5) gland may show retrograde metastasis via the lymphatic plexus in the subcutis of the inner thigh and to the popliteal lymph nodes. Retrograde metastatic spread is a rare event, but mammary carcinoma metastasis to the vagina have been reported in bitches [13, 14]. In normal healthy dogs, lymphatic fluids drain into the ipsilateral lymph nodes. There is no drainage to the contralateral gland or lymph node, but drainage may be altered in cases of mammary neoplasia [6].

Table 1 - Lymphatic drainage of normal and neoplastic canine mammary gland.

Mammary Gland	**Normal Lymphatic Drainage** [15, 16]	**Neaplastic Lymphatic Drainage** [12]
M1, cranial thoracic	Axilary LN	Axillary LN, Sternal LN
M2, caudal thoracic	Axillary LN	Axillary LN, Sternal LN
M3, cranial abdominal	Axillary LN, Superficial inguinal LN	Axillary LN, Superficial inguinal LN, Medial iliac LN
M4, caudal abdominal	Superficial inguinal LN	Superficial inguinal LN, Axillary LN
M5, inguinal	Superficial inguinal LN	Superficial inguinal LN, Popliteal LN

LN, lymph node.

Lymphatic vessels can also cross the abdominal midline and in doing so pass through the abdominal and thoracic walls. The axillary lymph node collects lymph from thoracic mammary glands, while the superficial inguinal lymph node collects lymph from the

abdominal mammary glands. Generally, the lymphatic drainage of the third mammary gland occurs cranially to the axillary lymph node, but deviation is possible to the inguinal drainage region [8, 11]. The dual drainage system of this mammary gland is explained by the formation of plexiform lymphatic connections in between the abdominal and caudal thoracic mammary glands [12, 15, 16].

Canine Mammary Tumors (CMT)

A General Approach

The investigations of mammary tumors in dog are of particular interest because they constitute an excellent model for the study of breast cancer in women. In fact mammary tumors are very common in the population of domestic dogs and show remarkable clinical, histopathological, and even genetic and molecular similarities with women breast cancer [4].

The recent sequencing of the canine genome and the evidence of its similarity to the human genome has emphasized the dog as an attractive alternative model for cancer research [17, 18]. In addition, dogs have a considerable body size, genetic variability similar to humans, and develop spontaneous tumors in the context of a natural immune system. Moreover, unlike laboratory animals, these species share the same environment and are exposed to the same carcinogens; however, they develop tumors in shorter time, due to their shorter longevity compared with the humans [4, 19-22]. In the future it's possible that this comparative oncology can play a role in translational medicine, making early clinical applications from knowledge obtained from this model [23].

In bitches, mammary tumors are the most common type of cancer, with a high incidence in geographic regions where ovariohysterectomy is not commonly practiced [24]. When both sexes are considered, breast tumors are the most common non-cutaneous tumors [25]. As in humans, there is a marked sexual predisposition, males have a risk of developing breast tumors equal or less than 1% [26, 27]. Studies conducted over the past decade reveal that in dogs diagnosed with cancer, 56% have mammary tumors [28] and the incidence rate is 205/100,000 cases per year [29].

Predisposing Factors

The incidence of CMT increases with age [30, 31], with middle-aged and older bitches mostly affected [32]. The mean age of incidence is approximately 10 years; however, the

risk of mammary tumors is increased in dogs over 6 years of age [33, 34]. Animals younger than 4 years are rarely affected by this condition [27]. The existence of a racial predisposition is a matter of disagreement among authors. Different breeds have been identified as predisposed to the development of these tumors: English springer spaniel, Doberman, Boxer [30], and also Cocker Spaniel, Dachshund [35] and Bouvier Bernois [36]. One study performed in 101 bitches reveals a lower incidence and a smaller dimension of malignant mammary tumors in small breeds [37]. This divergence between authors is possibly caused by the different contribution of each breed to the sample size under study. According to some researchers, obesity and type of diet influence the risk of developing mammary tumors in dogs, similar to what happens in humans [38]. In women, particularly during menopause, obesity is associated with increased development of breast cancer [24, 31, 39, 40] and epidemiological studies have shown that consumption of high fat diets is positively correlated with mortality rates caused by this type of cancer [41]. The low prevalence of mammary tumors in herbivores leaves us tempted to assume that a diet low in fat and high in vegetable fiber is beneficial. This is contrary to what happens in dogs, who feed mainly on meat (high fat and a tiny amount of fiber) and have a high prevalence of mammary cancer [42]. In dogs, obesity at a young age, as well as a diet based mostly on red meat and homemade food, appear to be factors potentially associated with a higher risk to develop mammary tumors and dysplasias [43].

Aetiology

The development of a neoplasia is a complex process resulting from various carcinogenic factors, such as ionizing radiation, chemical compounds and oncogenic viruses. The impact of several endogenous factors, genetic, immune and hormonal, is of irrefutable importance and deserves special attention [27]. Hormonal factors have been thoroughly studied and play a fundamental role in canine mammary carcinogenesis [44-46]. The development and growth of the mammary gland is influenced by ovarian hormones (e.g. estrogen and progesterone) and the functional contribution of these hormones has been acknowledged in various physiologic and pathologic situations [42]. As for the genesis of canine mammary gland neoplasia, the role of these hormones has been well established [42, 47, 48]. The first observation of the hormonal contribution in mammary carcinogenesis arose in the late 70s, when the effect of administering progestogen inhibitors of the oestrous cycle was first described [49]. This hypothesis is further supported by evidence of the protective effect of ovariohysterectomy (OVH) in the

development of mammary tumors, especially when it occurs at an early age, since the source of hormones is surgically removed [27, 31, 44]. It has been reported that mammary tumor incidence rises sharply whenever the OVH is performed following the second oestrous cycle. When compared with female dogs spayed before the first oestrous cycle, those spayed after the first and second oestrous cycle present an 8% and 26% increased risk of developing mammary tumors, respectively [50].

Analysis of steroid hormone participation in the acquisition of malignancy has shown a loss of estrogen receptors during the neoplastic progression, which suggests a hormone independence at stages of higher tumor aggressiveness [51]. However, conducting an OVH as a therapeutic measure after the initiation of cancer progression does not seem to have a significant beneficial effect in the course of the neoplastic disease [52]. In fact, CMT appear to be clearly dependent on steroid hormones (estrogen and progesterone) and therefore, the expression of estrogen and progesterone receptors constitutes additional evidence of the endocrine component in mammary neoplasia [53, 54].

Presently, other hormones are also known to be involved in mammary carcinogenesis. During CMT development, changes are found in the production of pituitary hormones, prolactin (PRL) [45] and growth hormone (GH), have a specific function in mammary tumorigenesis [46]. Malignant CMTs produce greater amounts of prolactin compared with benign CMT, and these also produce more prolactin that normal mammary glands. Prolactin and steroid hormones are produced in the mammary gland, functioning in an autocrine-paracrine mode as local growth factors, favouring the progression of malignant mammary tumors [45]. Concerning GH, excessive production induced by endogenous or exogenous progestagens might promote mammary tumorigenesis while stimulating the proliferation of susceptible mammary epithelium cells [48]. GH tissue levels in malignant CMT were also astoundingly augmented in relation to benign CMT [46]. Over the last decade, some studies point out the existence of other factors intervening in canine mammary carcinogenesis, such as insulin-like growth factor I (IGF-1) [46] epidermal growth factor (EGF) [55] and epidermal growth factor receptor (EGFR) [56].

Besides endocrine factors, genetic mutations are crucial in the development and progression of tumors. The first to occur is the initiation step, an initial lesion that will evolve under the influence of promoting factors until the later stages of invasion and metastasis unfold. Among several genes participating in mammary carcinogenesis, p53, p27 and c-erB-2 or HER2/neu are worth mentioning for the research conducted throughout the last decade [57-59]. The p53 gene is a major regulator of tumor

suppression, inducing the apoptosis of cells bearing damaged, irreparable DNA such as neoplastic cells. p53 gene mutations have been reported in 30-35% of CMT [57, 60, 61]. Not only do malignant and metastatic tumors bear p53 mutations, but also benign CMT, which might indicate this is an early event in mammary carcinogenesis [62].

The p27 gene, an inhibitor of cyclin dependent kinases, hampers the transition to the cell cycle replicative phase. Primary carcinomas and lymph node metastasis have a reduced expression of p27 mRNA, suggesting a preponderant role in tumorigenesis [58].

HER2/neu is an oncogene frequently mutated in human breast cancers, as the encoded receptor is targeted in clinical application by the drug Herceptin. The involvement of this gene as a prognostic marker in CMT is yet to be well established, but the expression of HER2/neu in tumor biopsies, coupled with other molecular markers (e.g. estrogen receptors, p63 and CK5) is useful in determining a molecular phenotype which, similarly to what has been observed in the human counterpart, presents a relationship with a worse prognosis [59]. The environmental aetiology has already been suggested, but hitherto not proven true. It is possible that anti-parasitic drugs containing pyrethroid substances accumulate in the peri-mammary adipose tissue and sensitize epithelial cells to proliferation [63].

Diagnosis and Clinical Management

CMTs may present as solitary or multiple masses. The caudal mammary glands are more affected than cranial glands, probably due to their larger size. Clinical signs that indicate a malignant lesion include the observation of the rapid growth of a mass not circumscribed, invasiveness, adherence to the skin or surrounding tissues, and ulceration. However the absence of these signs does not mean that it is excluded as malignant lesion [34]. Similarly, large lesions can merely mean that the search for veterinary care was delayed [24]. Mammary tumors are staged according to the World Health Organization (WHO) TNM system or a modification of this system [64, 65] (**Table 2**).

To stage the mammary gland tumors in a dog, information regarding tumor size, lymph node status, and presence of metastasis needs to be collected and verified. The largest malignant tumor in dogs with more than one tumor should be considered in the stage assignment. The lymph nodes should be identified, and if they are clinically enlarged, fine-needle aspirates for cytological evaluation must be performed. Dogs with malignant mammary tumors would also be evaluated for metastatic disease. Three-view thoracic radiographs remain the standard diagnostic method for evaluating veterinary patients for

the presence of metastasis [6, 24], since the lungs are the most common site for distant metastasis [66, 67]. However, additional staging tests, including abdominal ultrasound, skeletal radiographs, or other imaging modalities, may be indicated if clinical examination identifies other sites suspicious for metastasis. On physical examination, clinical signs suggestive of breast cancer are easily identified; however, definitive diagnosis of CMT is reached only after a histopathological study of the sample obtained by biopsy. The cytology by fine needle aspiration, despite being a minimally invasive methodology and easy to perform, is not reliable in the diagnosis of mammary tumors in female dogs due to the high variability and pleomorphism that the same histological tumor can present. Despite the limitations in diagnostic, cytology is a good method to dispose of some differential diagnoses and to determine the nodal disease [68].

Table 2 - Canine mammary tumor staging - modified WHO.

T: primary tumor T1: <3 cm maximum diameter T2: 3-5 cm maximum diameter T3: >5 cm maximum diameter	**Stages** **Stage I – T1, N0, M0** **Stage II – T2, N0, M0** **Stage III – T3, N0, M0** **Stage IV – Any T, N1, M0** **Stage V – Any T, Any N, M1**
N: regional lymph node status N0: no metastasis N1: metastasis	
M: distant metastasis M0: absent M1: present	

For neoplastic lesions of the mammary gland in dogs, the classification recommended by the World Health Organization (WHO), published in 1999, is commonly accepted [69]. The breast neoplasms are divided into benign or malignant, and they may have their origin in epithelial, myoepithelial or mesenchymal tissue. Most mammary gland tumors are of epithelial origin. Some, however, can have mixed histology consisting of both epithelial and myoepithelial tissue, with areas of cartilage and bone, and a few tumors are of purely mesenchymal origin [24]. The term inflammatory carcinoma is not included in WHO classification and is a distinct clinical entity in dogs, characterized histologically by the presence of emboli in dermal lymphatic vessels which is responsible for the severe

regional edema observed in most cases [70, 71]. Recently, a new proposed classification of canine mammary gland lesions covers the inflammatory carcinoma as a special kind of carcinoma [72].

During embryonic development of the mammary gland, there is a close relationship between the epithelium and mesenchyme. Therefore, in tumoral development of complex adenomas there are combinations of epithelial and myoepithelial components, with mixed mammary tumors presenting cartilage and bone [6]. CMT have a complex histogenesis which is not yet completely understood. Stem cells of ductolobular terminal units are identified as a possible origin of many dysplasic and tumoral lesions of the mammary gland [73]. The application of immunohistochemical techniques, creation of cell lines, and transplant assays has deepened the analysis of mammary tumors histogenesis. In complex adenomas and benign mixed tumors, there is a demonstrated involvement of epithelial and myoepithelial components, as shown from the immunohistochemistry of specific molecular markers CK5 and p63, respectively [74, 75].

In CMT, it is a relatively common occurrence to have multiple tumor masses. This fact has been overlooked as several different types of tumors are consistently considered as independent primary tumors, isolated and inaccessible to signalling from other tumors in the same organism. Recent studies have nevertheless pointed to an increased risk for the development of malignancies after a first diagnosis of mammary cancer [6]. Recent data also reveals that CMT begin as benign tumors and progress through successive histological types of aggression. This development culminates in the formation of malignant tumors, showing similarities with the process of carcinogenesis in human breast cancer [5]. The proportion of malignant CMT is 50% [76], and approximately 50% of breast carcinomas metastasize to regional lymph nodes and lungs [76, 77]. The risk of metastasis is influenced by tumor histological type, histological grade of malignancy and several prognostic factors.

Histological Classification of Canine Mammary Tumors: World Health Organization Guidelines

The most recent studies conducted in CMT have been based on the classification proposed by the World Health Organization (WHO) [46, 78, 79]. This classification system combines histogenic and descriptive morphologic parameters, also incorporating histologic prognostic features that have been associated with an increasing malignancy. **Table 3** presents all histological types and the reader is encouraged to consult the

bibliographic source where can find a detailed description of each tumor type. The most common types of malignant CMT are presented in **Figure 1** in an increasing order of malignancy.

Table 3 - Histological Classification – WHO.

Malignant tumors	**Benign tumors**
Non-infiltrating (in situ) carcinoma	**Adenomas**
Complex carcinoma	Simple adenoma
Simple carcinoma	Complex adenoma
Tubulopapillary carcinoma	Basaloid adenoma
Solid carcinoma	**Fibroadenoma**
Anaplastic carcinoma	High-cellularity fibroadenoma
Special types of carcinoma	Low-cellularity fibroadenoma
Spindle cell carcinoma	**Benign mixed tumor**
Squamous cell carcinoma	**Duct papilloma**
Mucinous carcinoma	
Lipid-rich carcinoma	
Sarcoma	
Fibrosarcoma	
Osteosarcoma	
Other sarcomas	
Carcinosarcomas	
Carcinomas or sarcomas in benign tumor	

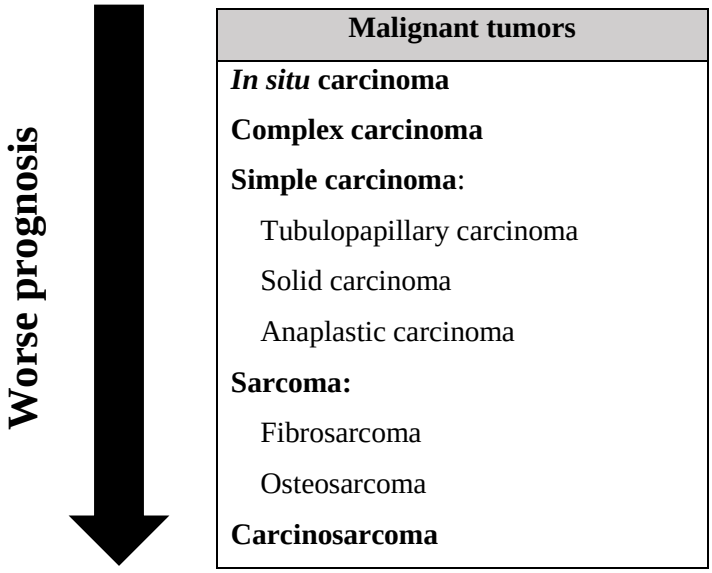

Figure 1 - Prognosis of malignant CMT.

Prognostic Factors

Due to the heterogeneity of pathological types and clinical presentations of CMT, the histological evidence of malignancy does not necessarily imply a malignant clinical course of the disease [80]; therefore, in veterinary medicine, the meticulous search and communication of a reliable prognostic factors in a short period of time is an urgent task for the veterinary clinician. A determined characteristic or feature of the dog or the tumor itself is considered as a prognostic factor when it is capable of providing reliable information that allows predicting the onset of recurrence and metastasis after clinical remission. For this reason, in prospective clinical studies, the characteristics with prognostic value should be evaluated based on their ability to predict disease-free survival (DFS, time from the date of the surgery till the occurrence of recurrence and/or metastasis) and overall survival time (ST, time from surgery until death of the animal) [6].

As mentioned previously, the presence of considerable variations in the biological behaviour, within series of histologically malignant mammary tumors, makes it impossible to obtain a precise prognosis, using no more than the microscopic histopathologic analysis [81]. The age of the animal at the time of surgery is associated with disease free survival and overall survival in some studies, where older animals show a lower survival rate when compared with young animals [24, 34, 82, 83]. The rapid and invasive growth rate of the tumor, as well as a larger size, are clinical parameters classically associated with a worse prognosis in dogs with malignant mammary tumors [73, 84-86].

The presence of tumor ulceration provides valuable information because it is a clinicopathological feature correlated significantly with tumor malignancy and poor survival [82, 86, 87]. The prognostic value of regional lymph node involvement at the time of surgery has been confirmed by several researchers [73, 82, 85, 86, 88, 89]. According to several researchers, mitotic index, nuclear grade, and distant metastasis show a statistically significant association with disease-free survival and/or overall survival, and are also considered as prognostic factors [86, 87].

Although in the context of CMT a range of clinical and pathological factors with reliable prognostic value have already been identified, it is evident that a complete understanding of tumor behavior is still years away. With the relentless pursuit of understanding this highly complex and dramatically incident phenomenon, the scientific community is

focusing on the molecular level to uncover how the tumor processes unfold. In addition, the identification of molecular markers allows for the identification of new therapeutic targets. Currently, molecular markers for CMT have been identified that have a prognostic sustained interest which include: PCNA, Ki67, p53, COX-2, EGFR, tumor-associated macrophages (MAC387), and tumor infiltrating lymphocytes (CD3, CD4, CD8) [20, 56, 78, 79, 85, 90, 91]. Ki67 and PCNA are cell proliferation markers and are correlated with tumor histological grade, nuclear grade, presence of metastasis, death by cancer, and shorter disease-free survival after surgery [85, 87, 92]. The nuclear expression of p53 protein, resulting from the mutation of the p53 gene, also showed CMT prognostic value in being correlated with shorter survival and increased tumor recurrence [20, 61].
In CMT, some studies demonstrated an association between COX-2 expression and angiogenesis, the development of distant metastasis, a poor prognosis, and shorter survival periods [90, 93, 94]. With respect to EGFR, there was a tendency toward a shorter disease-free survival and overall survival in dogs with positive EGFR expression [56]. Tumor-associated macrophages revealed a significant association with the decrease in overall survival [78]. In malignant CMT, abundant lymphocyte infiltrates are frequently found and animals with high proportions of $CD4^+$ and low $CD8^+$ T-cells have lower survival rates [91]. In addition, there is a tendency for a higher number of CD3+ tumor-infiltrating T-lymphocytes and a shorter overall survival. Interestingly, a statistically significant relationship was observed only for $CD3^+$ T-lymphocytes in the adnexal non-tumoral mammary gland, with a higher number of lymphocytes conferring a reduced overall survival [79]. **Table 4** summarizes the most significant CMT prognostic factors. Despite substantial scientific advances in the area of CMT prognosis, there remains an incessant need to expand our knowledge in order to better understand the biological behavior of these tumors.

Treatment

Given an animal with a breast tumor, the primary treatment of choice is still surgery [44], excluding cases of animals with inflammatory tumors of the mammary gland (such as inflammatory carcinoma), or distant metastasis [6, 70]. The selection of the type of surgery is dependent on the extent of the tumor. Lumpectomy or nodulectomy is recommended for superficial and non-adherent masses smaller than 0.5 cm, which generally have a benign behavior. For 1 cm or larger lesions located at a deeper level and with adhesions, regional or radical mastectomy is recommended [70]. A prospective

clinical study demonstrated that after regional mastectomy, 58% of cases had recurrence of a new tumor in the mammary gland remains of the ipsilateral mammary chain [95]. Strategies for treatments in addition to surgery have not been well-established yet [24], and the use of chemotherapy or radiotherapy is limited due to their high cost. It becomes necessary to distinguish those animals with breast tumors that may most effectively benefit from the application of these treatments. Although adjuvant chemotherapy is a standard treatment in human breast cancer, limited information exists about its effectiveness in breast tumors in dogs. Routine use in the treatment of these tumors is not recommended because of its ineffectiveness compared to other types of neoplasms [70]. Nevertheless, chemotherapy may be useful as an adjuvant therapy to the surgical excision of tumors with a probable metastasis or recurrence. There are some studies in CMT which indicate the relationship of certain types of chemotherapeutic agents (e.g. a combination of 5-fluoroucil, cyclophosphamide and doxorubicin) and an increase in survival of animals [96, 97]. Although the impact of these studies is relatively low since they are based on a small sample of animals, they are proof-in-principle that the use of adjuvant chemotherapy may be required in some cases. However, additional well-controlled studies must be performed in order to determine the most appropriate chemotherapeutic agents to treat malignant CMT. Regarding radiotherapy, the scarcity of studies is evident and the results reveal unattractive effects for this therapeutic modality [24].

Table 4 - Summary of CMT prognostic factors.

Good prognosis	Poor prognosis	Indifferent
• <3 cm in diameter • Well circumscribed • Lymph node: negative • Ulceration absent • Proliferation index Ki-67 low PCNA low • Low COX-2	• >3 cm in diameter • Invasive, ulcerated • Lymph node: positive • Proliferation index Ki-67 high PCNA high • High COX-2 • p53 gene mutation	• Breed • OVH at time of surgery • Type of surgery • Number of tumors • Glands involved

In CMT, exogenous hormonal treatment is indicated in tumors exhibiting estrogen, progesterone, or prolactin receptors [70]. In human medicine, the use of hormonal manipulation is a common practice. Tamoxifen Citrate is a selective inhibitor of estrogen receptors in the mammary gland and is largely used in human breast cancer treatment strategies. In dogs, due to the high risk of developing pyometra, it is recommended that

the administration of tamoxifen is limited to sterilized animals. Moreover, since there is no difference in side effects between higher and lower doses, clinicians should opt for the administration of the highest dose of the drug to increase the chances of therapeutic success [98]. Immunotherapy is at the forefront of therapeutic modalities to treat CMT. In one study, researchers used a vaccinal oncolytic virus (GLV-1h68) in the treatment of CMT. The virus was used to infect and kill canine mammary adenomas cell lines, as well as inhibit tumor proliferation and decrease tumor size in xenotransplants [99].

Recently, selective COX-2 inhibitors have been used in several studies to strengthen the possibility that these drugs could be used in a prophylactic or as adjuvant therapy in mammary tumors [100]. In dogs, clinical trials based on selective COX-2 inhibitors showed success in reducing clinical signs in transitional cell carcinoma of the bladder [101-103]. Recent studies in CMT revealed a relationship between COX-2 expression and more aggressive tumor phenotypes [104-107]. These findings emphasize the need for additional studies with selective COX-2 inhibitors in order to verify their true clinical effectiveness.

In canine inflammatory carcinomas, piroxicam (a nonselective inhibitor of COX-2) therapy showed an improvement of the clinical status *in vivo* [108] and significant cytotoxic effects were obtained *in vitro* using piroxicam and deracoxib against canine mammary carcinoma cells. This suggests that COX-2 inhibitors can be an attractive approach for the treatment of CMT [109]. The importance of COX-2 has been recognized by clinicians in human and veterinary medicine and the increasing use of COX-2 selective inhibitors. In human medicine, promising results are already evident in published studies based on case series and randomized clinical trials [110, 111]. Given the complexity of tumoral development, therapeutic modalities will be constantly updated and refined, which is essential in the evaluation of combined and customized treatments.

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I.2 A Role for T-lymphocytes in Human Breast Cancer and in Canine Mammary Tumors

The mammalian immune system comprises a coordinated and finely controlled series of interactions involving cells and molecules and has an essential role on species survival and adaptation all over the years [1]. The immune system has the important task of distinguishing between "self" and "non-self", providing protection against foreign pathogens, maintaining at the same time tolerance to self-antigens [2, 3].

Cancer is a progressive process that arises from a well-defined step where somatic cells acquire activating (oncogenes) or deactivating (tumor suppressor genes) mutations [4, 5]. All types of cancer are caused by the progressive and uncontrolled growth of transformed cells and the control of this disease requires the ablation and destruction of all the malignant cells without damaging the patient. To attain this assignment the own body has to distinguish between the cells of the tumor and other cellular counterparts [3, 6]. However, unfortunately many tumors continue to grow progressively and expand, which demonstrates that immune system is not always effective and fails on its protective role against tumor development [3, 7].

Decades of intensive investigation left clear that the interplay between immunity and cancer is complex [8]. One example of this high complexity is the phenomena of **"Cancer Immunoediting"**. Cancer cells constantly modulate the host antitumor immune response in a process called **immunoediting**. During this process the balance between antitumor and tumor-promoting immunity can be tilted to protect against the neoplasia development or, on the contrary, to support tumor growth. Immunoediting is characterized as a three-phase process including: **elimination phase** (immunosurveillance), **equilibrium phase** and **escape phase** [5]. Therefore, the immune system can release factors that promote neoplastic cells survivor, growth and invasion. Thus, paradoxically, immune system acts as an extrinsic tumor-suppressor, but can also promote cancer initiation, promotion, and progression [9].

Chronic Inflammation and Cancer

The role of chronic inflammation in cancer was first proposed by Rudolf Virchow in 1863, when he observed the presence of leucocytes in neoplastic tissues. Virchow

postulated that an inflammatory milieu promotes a cellular environment that drives the initiation and development of carcinogenesis (Reviewed in: [10, 11]).

Inflammatory responses play a crucial role at different stages of tumor development [12, 13]. Innate immune cells that infiltrate tumors participate in an extensive and dynamic crosstalk with cancer cells and some of the molecular events that mediate this dialog have been revealed [6, 14]. The most relevant molecular mechanisms include increased production of pro-inflammatory mediators, such as cytokines, chemokines, reactive oxygen intermediates, increased expression of oncogenes, COX-2 (cyclooxygenase-2), 5-LOX (5-lipoxygenase), MMPs (matrix metalloproteinases), and pro-inflammatory transcription factors such as NF-κB (nuclear factor κB), STAT3 (signal transducer and activator of transcription 3), AP-1 (activator protein 1) and HIF-1α (hypoxia-inducible factor 1α). These pro-inflammatory mediators potentiate tumor cell proliferation, transformation, metastasis, invasion, angiogenesis, chemoresistance and radioresistance [11, 15-17].

So, **why does inflammation potentiate cancer development rather than protect against it?** In fact, chronic inflammation is considered important in promotion of cellular proliferation and cancer progression by enhancing angiogenesis and tissue invasion [5, 13], releasing products that promote carcinogenesis in nearby cells and accelerating genetic mutations through a state of malignancy [7]. Finally, through cancer-derived products, immune and regulatory cells are recruited and the weak tumor antigenicity subverts immune cells in order to support cancer progression [5, 18].

Adaptive Immunity and Cancer Development: A Role for T-Lymphocytes

In neoplastic lesions, the role of infiltrating T-lymphocytes is often paradoxical. Despite the evidence that the responses of T-lymphocyte can destroy tumor cells "in situ", these responses appear to be frequently ineffective in the elimination of the established cancer [19, 20]. In fact, patients with cancer present a deficient immune response to tumor antigens. However this deficient immune response is clearly different from immunosuppression observed in patients receiving high doses of corticosteroids and/or chemotherapy. The term "immune dysfunction" seems the most appropriate to describe the changes observed. The mechanisms that support this "immune disorder" include **barriers that prevent recognition of the tumor** by immune cells and also **lymphocyte dysfunction** [21].

The **barriers that prevent recognition of the tumor** by immune cells include several mechanisms such as: sequestration of tumor associated antigens and major histocompatibility complex (MHC) molecules, loss of co-stimulatory molecules and other molecules required by cytotoxic T-cells. These mechanisms represent a barrier for the total elimination of tumor [19, 22, 23]. In respect to the **lymphocyte dysfunction** that seems to be present in cancer patients, a tumor-directed immune response involving cytotoxic $CD8^+$ T-cells, T helper 1 (Th1) cells, and natural killer (NK) cells appears to protect against tumor development and progression. Contrarily, the immune responses that involves B-cells, the activation of chronic humoral immunity and/or a T helper 2 (Th2) polarized response and polarized innate inflammatory cells in the tumor, can promote tumor development and progression. This balance between a protective cytotoxic response and a harmful humoral or Th2 response can be regulated systemically by the general immune status of the individual [20, 24].

In this context, the question that arises is: **What is the reason why the responses mediated by $CD8^+$ cytotoxic T-lymphocytes are not effective in eradicating the tumor and how can the $CD4^+$ T-cells be involved in neoplastic progression of this disease?**

A part of the response has already been described above and is related to tumor escape mechanisms from cytotoxic $CD8^+$ T-cells action. Another important mechanism appears to be related to the polarity of the responses of $CD4^+$ T-cells in relation to the primary site of cancer and/or their distant metastasis [24] and the imbalance of the normal ratio of Th1/Th2 cells [25].

$CD4^+$ T-cells are activated in response to soluble factors and can be classified into categories, Th1 and Th2. After stimulation, the Th1 cells secrete interferon gamma (IFNγ), transforming growth factor beta (TGFβ), tumor necrosis factor (TNF) and interleukin 2 (IL-2). These cytokines cooperate with the functions of cytotoxic $CD8^+$ T-cells, producing a tumoricidal activity. In contrast, Th2 cells express interleukin (IL) 4, 5, 6, 10 and 13 that induce anergy of T-cells and loss of cytotoxicity, while increasing the humoral immunity (lymphocyte B function). Thus Th1 cell responses benefit anti-tumor immunity, whereas Th2 cell responses produce a down-regulation of anti-tumor cell mediated immunity and increase the humoral pro-tumorigenic responses [24, 26, 27].

Although the immune dysfunction in patients with cancer is now better understood with the perception of Th1 and Th2 regulation, what is responsible for this dysfunction remains to be determined. One possibility is that the number of Th1 cells or their precursors are

reduced, decreasing one line of defense against cancer progression and metastasis. Another possibility is the important role played by **regulatory T-cells** and **immature myeloid cells** in the anti-tumor immune suppression observed in patients with breast cancer and other type of neoplasms [25, 28].

Regulatory T-cells (Treg cells) are a distinct group of lymphocytes with immunosuppressive properties that usually maintain immune tolerance [29]. Treg cell suppressive activity is beneficial by restricting T-cell response against self-antigens and preventing inflammatory and autoimmune diseases. In cancer, their inhibitory role in limiting immune response against "pseudo self-antigens" from tumor origin avoids an effective anti-tumoral immune response and often culminates into negative outcomes for the patient. These cells may play an important deleterious role in cancer immunopathology due to their potent suppressive activity of both T-cell activation and effector functions [20, 30, 31].

Immature myeloid cells express MHC class I molecules suggesting that they can induce cytotoxic T-cells anergy by binding to T-cell receptor (TCR) complex in absence of co-stimulatory signals [32, 33].

In last years a new subset of $CD4^+$ (helper) T-cells, termed **Th17 cells**, has been characterized. Th17 subset secretes IL-17, IL-21, IL-22 and play critical roles in the pathogenesis of inflammatory and autoimmune diseases, as well as in host protection against pathogens. Although some data suggest the importance of Th17 cells for tumor immunity, conclusions regarding the functional role of Th17 cells remain controversial [34-37]. Even though some studies indicate that mouse Th17 cells support a positive anti-cancer immunity, the Th17 cells with intra-tumoral location are probably responsible for chronic tissue inflammation and appear to have a tumor-promoting effect [35, 38, 39].

T-Lymphocytes and Human Breast Cancer: Friends or Foes?

In humans, the study of the inflammatory infiltrate, mainly T-lymphocytes, has been subject of great interest associated not only with breast cancer [19, 23, 40], but also with other types of neoplasias, including seminoma [41, 42], melanoma [43, 44], colorectal [45, 46], cervical [47], ovarian [48, 49], urothelial [50] and gastric cancer [51].

In breast cancer an important role has been attributed to inflammatory cells, as well as cytokines produced by them. A large number of observations suggest that inflammatory cells are not "innocent spectators", but contrarily, they might conspire with the tumor cells favoring tumor development and progression [8]. However, the prognostic

significance of infiltrating T-lymphocytes is still subject to considerable debate [24, 52], because no definitive conclusions have been reached so far. The T-lymphocytes infiltrate appear, according to some researchers, associated with a better prognosis, whereas in other cases is related to a decline in overall survival. **Table 5** illustrates some of the most relevant studies in this area in the last two decades [19, 25, 53-69].

More recently investigations that focus on understanding the functions of Treg cells and Th17 cells in mammary carcinogenesis have been published, however the results of the various studies are also quite controversial [70-72].

Lee and collaborators [70] investigated, by immunohistochemistry, whether the presence of $FoxP3^+$ Treg cells was associated with prognostic factors, such as stage or histologic grade. $FoxP3^+$ Treg cells were, in this study, an independent prognostic factor for overall survival and progression free survival. The improved survival times were associated with highly infiltrating $FoxP3^+$ Treg cells.

Another study, on the contrary, showed that the increased number of Foxp3 Treg cells was significantly correlated with lymph node metastasis and immunopositivity for Ki-67, which indicates a probable relationship with a worse prognosis. [71].

Wang and colleagues assessed the Th17 and Treg cells by flow cytometry and observed that Th17 and Treg cells accumulation in the tumor microenvironment of breast cancer occurred in early stages of disease. With tumor progression, Th17 cell infiltration gradually decreased and there was accumulation of Treg cells [72]. So, this last study indicates that an increase in the number of Treg cells is associated with tumor progression.

The apparent controversy among distinct studies emphasizes the need for further research on this topic. A clear understanding about the role of T-lymphocytes in breast cancer is essential for the development of new therapeutic strategies in a near future.

T-Lymphocyte Infiltrate in Canine Mammary Tumors

Canine mammary tumors are a spontaneous neoplasia that occurs frequently in the clinical practice [73, 74]. Although the high number of studies published on this subject in the last decades, little is known about the role of tumor inflammatory infiltrate in cancer development and/or progression. In dogs, the first studies focused on inflammation and cancer, have been performed in other type of tumors including transmissible venereal sarcoma [75], benign oral papilloma [76], cutaneous histiocytoma [77] and seminomas [78]. Studies investigating the role of inflammation in canine mammary tumors were only recently published (**Table 6**) [79-84].

In canine transmissible venereal sarcoma, the quantity of T-lymphocytes is higher in the group of tumors that exhibit spontaneous regression or stable growth, comparatively with the tumors that exhibit a progressive growth [75]. In canine oral papilloma, similar to humans [85], the maximum number of T-cells that infiltrate the tumor occurs during rapid tumor regression [76]. In canine cutaneous histiocytoma, in the same way, a lymphocytic infiltrate represents the morphological expression of one anti-tumor immune response, which correlates with observations of spontaneous regression *in vivo* [77, 86]. In turn, in canine seminomas [78], in accordance with what occurs in human seminomas [41, 42] infiltrating lymphoid cells consist mainly in T-lymphocytes, especially $CD8^+$ cells, which means that the reaction of the body against neoplastic cells is mainly cytotoxic. This, together with the number of MHC I positive cells and a high amount of antigen presenting cells observed, suggests, according to the authors, that inflammatory cells exhibit a role in anti-tumor response [78]. This might explain the biological behavior of these tumors that rarely metastasize and the favorable prognosis that often presents. Interestingly in 2007, Horiuchi and collaborators [87] refer that in animals with cancer, a smaller amount of Th1 cells and a significant larger amount of Th2 cells, compared to healthy ones, was observed. Considering that Th2 cells have an action that promotes tumor progression, these results have come refute what is already known in human works and relaunch the interest in this subject in dogs.

In canine mammary tumors, as previously stated, there are only a very limited number of studies, all of them recently published, that focus on effect of T-lymphocyte infiltrates and malignancy [79-84]. In malignant mammary tumors abundant lymphocyte infiltrates are frequently found, but the characteristics associated with lymphocyte infiltration in these tumors remain largely unknown. As in humans the controversy among distinct reports remains an important issue to be clarified (Table 6). According to Estrela-Lima and collaborators [79], lymphocytes represent the predominant cell type in the tumor infiltrate. The relative percentage of $CD4^+$ T-cells was significantly greater in metastasized tumors, while the percentage of $CD8^+$ T-cells was higher in cases without metastasis. Consequently, the $CD4^+/CD8^+$ ratio was significantly increased in cases with metastasis and was associated to lower survival rates. Authors defend that the intensity of lymphocytic infiltrate and the $CD4^+/CD8^+$ ratio may represent important survival prognostic biomarkers for canine mammary carcinomas.

In one study performed by Kim and colleagues [80], immunohistochemistry, immunoblotting and reverse transcriptase-polymerase chain reaction were used to evaluate tumor infiltrating lymphocytes (TILs) and the presence of related cytokines, as well as the expression of breast cancer susceptibility gene-1 (BRCA1). The results of this study revealed a correlation between expression of interleukin (IL)-1 and IL-6 and tumor metastasis. An association among the expression of TILs, cytokines and mutation of BRCA1, was also verified, suggesting that all of these factors may play a role in tumor progression.

In another study developed by our team [81], $CD3^+$ T-lymphocytes were evaluated in three distinct areas: within the tumor, in the periphery of the tumor and in the adnexal non-tumoral mammary gland. We observed a tendency towards an association of a higher number of $CD3^+$ tumor-infiltrating T-lymphocytes and a shorter overall survival. However, interestingly, only for $CD3^+$ T-lymphocytes in the adnexal non-tumoral mammary gland, a statistically significant relationship was observed, with a higher number of lymphocytes conferring a reduced overall survival. This could indicate that $CD3^+$ T-lymphocytes in adnexal non-tumoral mammary gland were implicated in tumor progression and survival, showing that its pro-tumorigenic immune responses may somehow be the starting point for the growth and progression of tumor cells.

Saeki and collaborators [82] assessed the number of tumor infiltrating T-lymphocytes, B-lymphocytes and antigen presenting cells by immunohistochemistry. As a result, the authors found a statistically significant increase in the number of intratumoral T-lymphocytes in malignant tumors compared with benign ones. The results of this study indicate a positive relationship between a high number of TILs and increased canine mammary tumors malignancy.

Very recently Kim and colleagues [84] demonstrated, by immunohistochemistry, that the degree of lymphocyte infiltration was significantly higher in canine mammary carcinomas with lymphatic invasion and high histologic grade, suggesting the importance of lymphocytes on tumor aggressiveness and greater malignant behavior.

Treg cells, whose activity is closely associated with the transcription factor FoxP3, have a suppressive action on T-lymphocytes anti-tumor responses [88, 89]. In dog there are few studies that focus on the action of Treg cells in tumor development and progression [83, 90-92]. Dogs with cancer had increased numbers of Treg cells in their peripheral blood and tumor-draining lymph nodes compared to healthy animals [90].

In dog mammary tumors, a recent study by Kim and Colleagues [83], described abundant Treg cells associated with high histological grade and lymphatic invasion. The number of Treg cells infiltrating intratumoral areas was markedly increased in tumors with poor prognostic factors, such as high histological grade of malignancy, presence of lymphatic invasion and presence of tumoral necrosis. These findings suggest that Treg cells might play a key role in canine mammary tumors progression. Furthermore the amount of intratumoral Treg cells may provide a new prognostic factor when assessing survival times, which may in turn lead to the development of new immunologic therapies.

The studies described above concerning canine mammary tumors, describe results that are in agreement with those from studies already published in human breast cancer which may be an indication of similar cancer immunologic aspects between the two species (**Figure 2**).

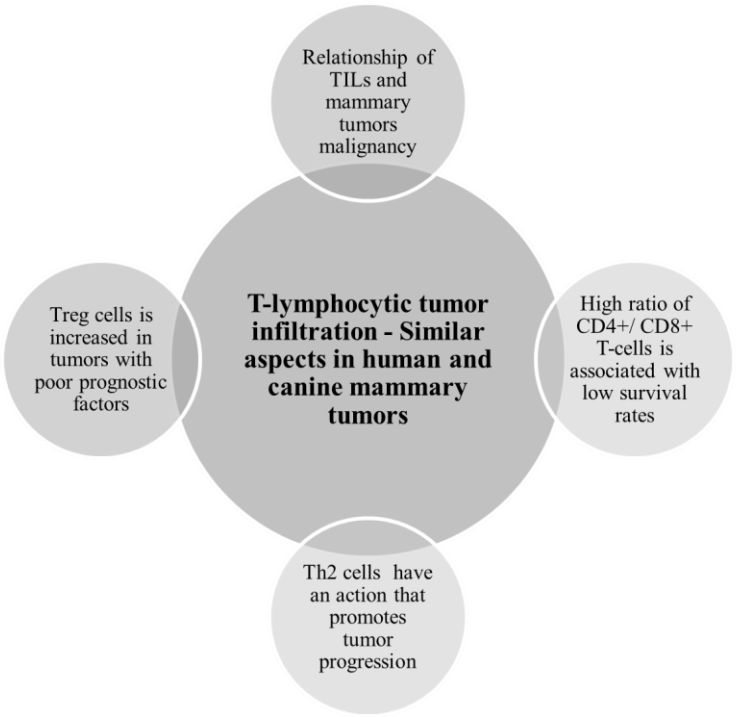

Figure 2 - Similarities between human breast cancer and canine mammary tumors regarding tumor T-lymphocyte infiltration.

Table 5 - Studies of a generalized lymphocytic infiltration in human breast cancer.

Author	Year	Patients n	Analysis	Location	Type	Comments
Aaltomaa et al.[53]	1992	489	Semiquantitative (none/mild–moderate–severe)	Peri-tumoral Intra-tumoral	Lymphocytic infiltration	Better prognosis, positive correlation with the lack of regional lymph nodes involvement, with a smaller diameter of the tumor, with a lower histologic grade and with a larger time free of disease.
Carlomagno et al.[54]	1995	1257	Semiquantitative (absent / present)	Peri-tumoral	Lympho-plasmacytic infiltration	Poorer overall survival in multivariate survival analysis in particularly patients with grade I and II.
Menard et al.[55]	1997	1919	Semiquantitative (absent / present)	Intra-tumoral	Lymphoid infiltration	Better overall survival in patients <40 years of age, no significant association in patients >40 in multivariate survival analysis
Marsigliante et al.[56]	1999	90	Quantitative (Computerized counting)	Intra-tumoral	T-lymphocyte infiltration	$CD3^+$ TILs was directly correlated to age, lymph node negative patients had tumors infiltrated by fewer $CD4^+$ TILs with respect to lymph node positive patients.
Georgiannos et al.[57]	2003	60	Semiquantitative (0 = none; 1 = rare cells; 2 = moderate numbers; and 3 = many positive cells)	Intra-tumoral	Lymphocytic infiltration	A significant association was found between the intensity of TIL and the number of positive nodes.

Table 5 - Continued

Author	Year	Patients n	Analysis	Location	Type	Comments
Campbell et al.[25]	2005	84 patients 26 healthy volunteers	The flow cytometry analysis was performed using CELLQuest software (BD Biosciences)	Peripheral blood and bone marrow aspirates	Peripheral blood lymphocytes	The percentages of both $CD4^+$ and $CD8^+$ cells were significantly lower in patients with breast cancer compared to healthy controls. Was observed a correlation between number of micrometastases in the bone marrow and T cell responsiveness.
Lee et al.[58]	2006	679	Semiquantitative (none–mild–moderate– severe)	Peri-tumoral Intra-tumoral	General inflammatory infiltrate	Better recurrence-free survival and overall survival in multivariate survival analysis.
Macchetti et al.[19]	2006	23	Flow cytometry quantitative analysis	Intra-tumoral	Lymphocytic infiltration	In the patients with lymph node metastasis, an increased mean percentage of tumor infiltrating $CD4^+$ T-cells, but not $CD8^+$ T-cells was observed and was correlated with worse prognosis.
Al Murri et al.[59]	2008	168	Quantitative analysis	Peri-tumoral Intra-tumoral	CD4 and CD8	No significant association.
Calabro et al.[60]	2009	155	Quantitative analysis microarray based screening for Li-associated Genes		Lymphocytic infiltration	Poorer overall survival in ER^+ patients and better overall survival in ER^- patients.

Table 5 - Continued

Author	Year	Patients n	Analysis	Location	Type	Comments
Rakha et al.[61]	2009	1597	Semiquantitative (mild - severe)	Peritumoral Intra-tumoral	General inflammation	Better recurrence-free survival and overall survival.
Rody et al.[62]	2009	1263	Quantitative analysis	Peri-tumoral Intra-tumoral	CD3	Better recurrence free survival in cases who had HER-2^{+}.
Matkowski et al.[63]	2009	88	Semiquantitative (percentage of positive stained cells: 0=none, 1=up to 33%, 2=33-66%, 3=more than 66%; intensity of the lymphocytic infiltrate: 1-low, 2- moderate, 3 - high)	Intra-tumoral	Lymphocytic infiltration	In early breast cancer the presence of CD8^{+} and CD4^{+} cells was correlated with lymph node involvement and unfavorable prognosis.
Baker et al.[64]	2011	1953	Quantitative analysis (TMA)	Peri-tumoral Intra-tumoral	CD8	Better cancer-specific survival in only high grade ER^{-} tumor in multivariate survival analysis whereas poorer cancer specific survival in low grade ER^{+} tumor in univariate analysis.
Ladoire et al.[65]	2011	162	Semiquantitative (none–mild–moderate–severe)	Peri-tumoral Intra-tumoral	CD8	Better recurrence-free survival after chemotherapy. CD8/FoxP3 ratio independent predictive factor associated with improved recurrence-free and overall survival after chemotherapy.

Table 5 - Continued

Author	Year	Patients n	Analysis	Location	Type	Comments
Liu et al.[66]	2011	1270	Quantitative analysis	Peri-tumoral Intra-tumoral	CD8	No significant association.
Mahmoud et al.[67]	2011	1334	Quantitative analysis (TMA)	Peri-tumoral Intra-tumoral	CD8	Better cancer-specific survival in multivariate survival analysis. Better recurrence free survival and cancer specific survival in only ER^- in univariate survival analysis. Whereas no significant association in ER^+.
West et al.[68]	2011	255	Quantitative analysis microarray based on information in the BioGPS gene portal	Intra-tumoral	Lymphocytic infiltration	TIL that express cytotoxic markers was strongly associated with favorable outcome after anthracycline based treatment of ER^- breast cancer patients.
Ruffell et al.[69]	2012	20	Flow cytometry, immunohistochemistry and confocal immunofluorescence quantitative analysis	Intra-tumoral	Leukocyte infiltration	Tumors from breast cancer patients treated with neoadjuvant chemotherapy contained an increased CD8/CD4 T-cell ratio compared with tumors removed from patients treated primarily by surgery alone.

Table 6 - Studies of T-lymphocytic infiltrate in Canine mammary tumors.

Author	Year	Patients n	Type	Comments
Estela-Lima et al.[79]	2010	51	T-lymphocyte infiltration	Animals with high proportions of $CD4^+$ and low $CD8^+$ T-cells had lower survival rates.
Kim et al.[80]	2010	58	T-lymphocyte infiltration	Association between the expression of TILs, cytokines and mutation of BRCA1, suggests that all of these factors may play a role in tumor progression.
Carvalho et al.[81]	2011	57	T-lymphocyte infiltration	Tendency for an association of a higher number of $CD3^+$ TILs and a shorter overall survival. $CD3^+$ T-lymphocytes in the adnexal non-tumoral mammary gland, revealed a statistically significant relationship with overall survival.
Saeki et al.[82]	2012	140	Lymphocytic infiltration	Relationship of TILs and canine mammary tumors malignancy.
Kim et al. [83]	2012	37	Regulatory T-cells (Treg)	The number of Treg cells is increased in tumors with poor prognostic factors, such as high histological grade, lymphatic invasion, and necrosis.
Kim et al.[84]	2013	47	Lymphocytic infiltration	Intense lymphocyte infiltration was associated with aggressive histologic features (higher histologic grade; lymphatic invasion).

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I.3 A Comparative Approach of Tumor Associated Inflammation in Mammary Cancer between Humans and Dogs

Most of the knowledge on tumor biology is derived from human and rodent studies. However, systematic comparison to spontaneous tumors in canine cancer patients could not only contribute to the improved understanding of the disease, but also to "translating clinical trials from human to veterinary oncology and back" [1]. This review aimed to compare, in both species, the relevant findings on the role of the immune regulation in mammary cancer.

Tumors are recognized as organs with high complexity and infiltrating immune cells are increasingly accepted to be generic constituents of them [2, 3]. Solid cancers often show signs of inflammation and are infiltrated by many leukocyte populations including T-lymphocytes and macrophages [4, 5]. The role of inflammation in carcinogenesis is not new. Over a century ago, a causal relationship between chronic inflammation and cancer formation was proposed taking into consideration the observations that cancers frequently develop at sites of chronic inflammation [6-8].

Chronic inflammatory responses associated with tumor sites have a multifaceted role in carcinogenesis. Indeed, it contributes to the acquisition of different hallmark capabilities by incipient neoplasms. Inflammatory cells can induce genomic instability, alterations in epigenetic events and consequent inappropriate gene expression [9, 10]. Furthermore, immune cells can provide bioactive molecules to the tumor microenvironment, including: i) growth factors that sustain proliferative signaling; ii) survival factors that limit cell death; iii) proangiogenic factors and extracellular matrix-modifying enzymes facilitating angiogenesis, invasion, and metastasis; and iv) inductive signals that lead to activation of other hallmark-facilitating programs [10-13].

During tumor progression, changes in tumor microenvironment induce a switch in innate immune cells toward a pro-tumorigenic function and actively contribute to immune tolerance, preventing rejection of the tumor by the immune system [2, 14]. In this process, dendritic cells (DCs) maturation can be suppressed by changes in the cytokine balance (increased VEGF, TGFβ, IL-10, IL-6, COX-2 and reduced IL-4, IL-12, IFN-α and IFN-γ) within the tumor, which significantly impairs the antigen presenting function of these cells [8].

Mammary cancer remains a major clinical challenge with considerable mortality both in humans and dogs [15-18]. Over the years, research on the association between inflammation and mammary cancer pathogenesis has blossomed in both species [19-22]. Alterations of the inflammatory components within the tumor microenvironment have a significant role during carcinogenesis and recent studies have highlighted the importance of tumor-associated immune cells and their influence on neoplastic progression [17, 23-26].

This review summarizes the intertwined signaling pathways shared by T-lymphocytic/macrophage infiltrates and important tissue biomarkers in both human breast cancer and canine mammary tumors (all the contents are synthetized in **Table 7** and illustrated in **Figure 3**). The inflammatory responses in mammary cancer sites are able to orchestrate hallmark-facilitating programs in the tumor microenvironment [27]. These phenomena could be important for prognosis as well as for the development of therapies aimed at redirecting the immune cells actions toward tumor destruction.

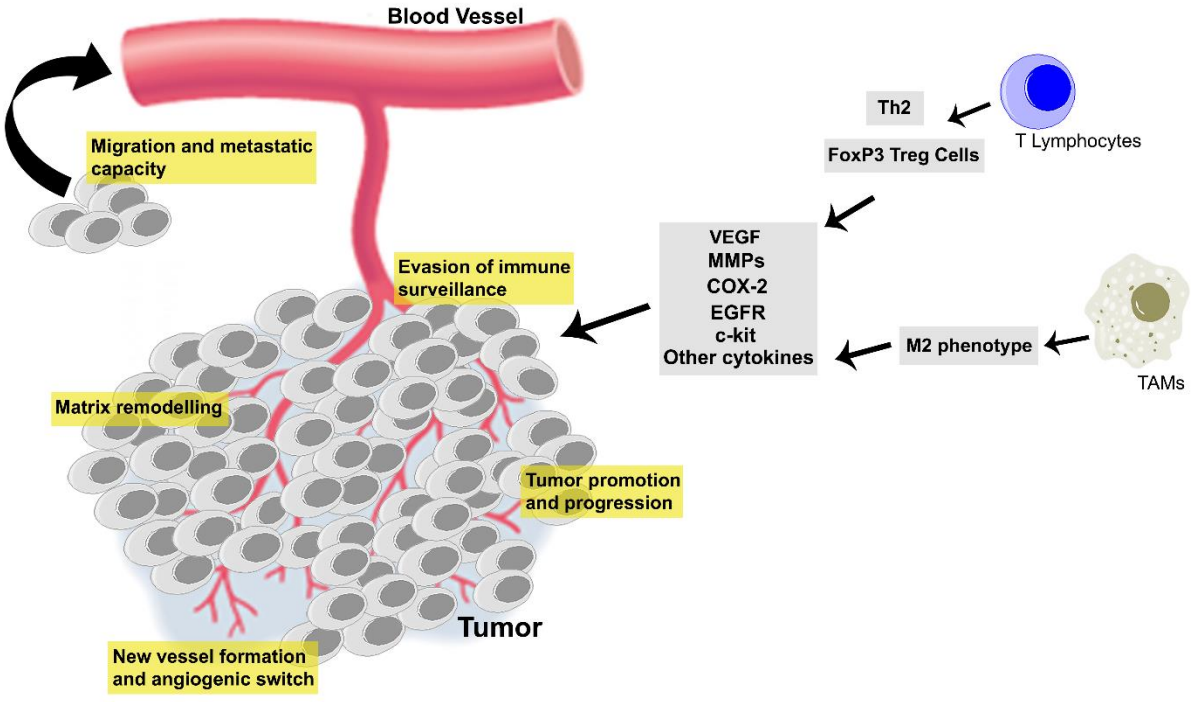

Figure 3 - Cancer related inflammation has an important role in mammary carcinogenesis, contributing to tumor evasion of immune surveillance, matrix remodeling and angiogenic switch, acquisition of metastatic capacity and tumor proliferation and progression.

Inflammatory Cells and Sustained Angiogenesis

Similar to normal tissues, vasculature has an important role in tumor growth and progression since it provides the necessary oxygen and nutrients that are present in the blood and allows discarding the toxic waste-products of metabolism [28, 29]. The tumor vasculature, driven by angiogenesis, is thus crucial for growth and survival of tumor cells, but it may also be exploited as a target for cancer therapy [30, 31]. The association between angiogenesis and mammary tumors has been a common subject of interest in humans and dogs [32-34]. In fact, human breast cancer progression is associated with robust angiogenic activity [35]. In highly metastatic human breast cancer there is an upregulation in the expression of pro-angiogenic factors, such as VEGF [36]. Similarly, in veterinary medicine, the presence of proliferating endothelial cells and blood vessels in intratumoral and peritumoral regions of benign and malignant canine mammary tumors was shown, with the peritumoral regions having the highest blood vessel area and perimeter. Furthermore, malignant tumors have significantly more new vessels and proliferating endothelial cells compared with benign tumors and normal mammary gland tissues [32]. We have shown previously that high microvessel density in canine mammary tumors was significantly correlated with tubule formation, with the histological grade of malignancy and with clinical stage [37]. Infiltrating immune/inflammatory cells secrete a diverse repertoire of pro-inflammatory mediators, such as cytokines, chemokines, growth factors and prostaglandins, among others, that further trigger inflammatory signaling thus attracting more inflammatory immune cells to the tumor microenvironment. Furthermore, some of these pro-inflammatory mediators directly stimulate the migration and proliferation of endothelial cells, thus promoting angiogenesis and consequently tumor growth [10, 38]. T-cells have an important role in the initiation and progression of inflammation by secreting a large number of cytokines and chemokines [39]. It was shown in *in vitro* studies that T-cells in inflammatory sites are in intimate contact with endothelial cells and influence angiogenesis. In fact, T-cells can secrete VEGF by specific antigens stimulation or by IL-2 and by hypoxia [39]. The human invasive breast carcinoma had a higher FoxP3 expression compared to the ductal carcinoma in situ and the adjacent normal tissues. Intratumoral levels of FoxP3, TGFβ1 and VEGF were found to be positively correlated to each other [40]. TGFβ1, by T-cell receptor (TCR) stimulation, induces FoxP3 expression in naive $CD4^{+}CD25^{-}FoxP3^{-}$ T cells and converts them into $FoxP3^{+}$ regulatory

T-cells. Furthermore, Treg cells enhance the TGFβ1 effects thereby creating a positive auto-regulatory loop of TGFβ1 signaling in $CD4^+CD25^-$ T-cells that potentially stabilizes their regulatory phenotype [41, 42]. The TGFβ1 and FoxP3 shared pathways induce an upregulated expression of VEGF which increase cancer vascularity and progression [40]. DCs are immunomodulatory cells that initiate adaptive immune responses, and exert pro-angiogenic effects in the tumor microenvironment. Immature DCs promote angiogenesis and tumor growth, whereas mature DCs are known to suppress angiogenesis. Using a mouse model of human breast carcinoma it was observed that rapid tumor growth is associated with the infiltration of immature DCs [43]. Tumor-derived VEGF-A is the main angiogenic factor that prevents DCs maturation by inhibiting the activation of the NF-κB via VEGFR-1 signaling [44]. Furthermore, another study reported that in human breast cancer DCs are differentiated into a phenotype that induce the expansion of Tregs by the expression of IL-10 and TGFβ1 [45], and the latter is a factor that indirectly induces the expression of VEGF [40]. Monocytes which become tumor-associated macrophages (TAMs) when entering the tumor also act as pro-angiogenic in human breast cancer [46]. In fact, the tumor microenvironment polarizes macrophages toward the M2 phenotype, which is characterized by elevated expression of potent pro-angiogenic factors. A transgenic mouse susceptible to mammary cancer confirmed that both the angiogenic switch and the progression to malignancy are regulated by infiltrated macrophages in the primary mammary tumors [47]. TAMs are thus responsible for the production of VEGF, of urokinase-type plasminogen activator (uPA) and of matrix metalloproteinase (MMP)-9 in human breast carcinomas [46, 48]. Additionally, some studies showed that the number of macrophages present in tumor stroma directly correlates with increased microvessel density, tumor size and cell proliferation in human breast cancer [49, 50]. Specifically, the infiltration of TAMs that express the chemokine CCL18 was positively associated with microvessel density in breast cancer. In fact, the synergistic expression of CCL18 and VEGF by the TAMs promoted endothelial cell migration and angiogenesis in this type of cancer [51].

Some studies from veterinary oncology report the implication of the pro-inflammatory mediators produced by the tumor-infiltrated inflammatory cells in canine mammary tumors progression and angiogenesis [52-55]. In accordance, we show in canine mammary tumors a positive correlation between infiltrating $CD3^+$ T-cells, VEGF and microvessel density, implying that $CD3^+$ T-lymphocyte cytokines in this type of cancer may stimulate angiogenesis through the induction of the pro-angiogenic factor VEGF.

Additionally, this high CD3/VEGF expression was associated with an elevated grade of malignancy, presence of neoplastic intravascular emboli, presence of lymph node metastasis and poor prognosis [52]. It was further shown that myeloid-derived suppressor cells (MDSCs) were significantly increased in stage III and IV canine mammary tumors and in this case MDSCs had significantly altered molecular pathways expressing amplified activation of IL-28/IL-28RA (IFN-γ) signaling. Moreover, IL-28 secreted by the MDSCs stimulates the STAT3 pathway which increases VEGF-C expression and therefore induces angiogenesis [56]. A positive correlation between microvessel density and mast cells in malignant canine mammary tumors was found, which, in turn, suggests that these immune cells play an important role in canine mammary cancer angiogenesis, similar to human breast cancer [53]. TAMs in canine mammary tumors have already been associated, by our group, with skin ulceration, histological type, nuclear grade, tubular differentiation and decrease in overall survival [57]. Similarly to human breast cancer, in canine mammary tumors the macrophages also polarize toward the M2 phenotype [58]. Recently, we and others demonstrated that TAMs infiltration is significantly associated with VEGF expression in malignant canine mammary tumors and that genes involved in angiogenic cellular pathways have been significantly upregulated in macrophages co-cultured with canine mammary tumor cell lines [55, 59].

Inflammatory Cells, Tissue Invasion and Metastasis

Cancer, in its most aggressive form, is not only a disease characterized by uncontrolled cell proliferation and growth but also by uncontrolled cell migration. The activation of angiogenic vasculature is very important for cells to amplify locally (i.e. malignant transformation) and/or spread systemically (i.e. metastasize) [60]. Cancer metastasis involves a complex sequence of processes starting with local invasion, followed by entry of the cancer cells into blood or lymphatic vessels, extravasation in distant tissues, formation of micrometastases, followed then by progression into macroscopic tumors [29].

The crosstalk between cancer cells and other cells present in tumor microenvironment is decisive for invasive growth and metastasis. In fact, tumor invasion and metastasis can be potentiated by an inflammatory infiltrate in tumor sites [61]. In addition to promoting angiogenesis, inflammatory infiltrates promote metastatic dissemination by enhancing migratory/invasive potential of neoplastic cells through the production of tissue remodeling proteases, cytokines and growth factors [62, 63]. Human breast cancer

development is characterized by a significant increase in lymphocytes in the neoplastic stroma [64]. For instance, Th2-polarized $CD4^+$ T-lymphocytes that express IL-4 promote invasion and subsequent metastasis of mammary adenocarcinomas by regulating the polarization and effector function of TAMs. In turn, M2-TAMs enhance metastasis through activation of EGFR signaling in human malignant mammary epithelial cells [64]. In mice bearing mammary tumors it was demonstrated that IL-1β elicits IL-17 expression by gamma delta T-cells, resulting in expansion and polarization of neutrophils by granulocyte colony-stimulating factor (G-CSF) action. Tumor-induced neutrophils acquire the ability to suppress cytotoxic $CD8^+$ T-lymphocytes, which favors the metastatic spread [65].

Hence, even the recruitment of monocytes to the tumor has an important role. In fact, CCL2 synthesized by metastatic tumor cells is critical for recruitment of a sub-population of CCR2 expressing monocytes that enhance the subsequent cell survival and extravasation through VEGF and M-CSF production [66].

Multiple studies in human cancers, including breast cancer, have reported that the presence of TAMs correlates with aggressive disease and outcome [67]. Structural changes in the extracellular matrix are necessary for cell migration. The proteolytic activities of MMP-2 and 9, expressed by macrophages, promote the release of cryptic fragments by cleaving laminin-5 γ2 chains which mimic EGF ligand and induce cell motility and invasion in EGFR overexpressing human breast carcinoma cell lines [46, 68]. The co-culture with M1 and M2 macrophages increased migration of ER-positive breast cancer cell lines [69]. In a study using a mouse model for breast cancer it was demonstrated that, in mammary tumors, CSF1 may promote metastatic potential by regulating the infiltration and function of TAMs [70]. In fact, using a metastatic breast cancer model it was possible to comprehend that the paracrine loop signaling between TAMs, which supply EGF, and breast cancer cells, which on the other hand supply CSF1, is sufficient for the promotion of invasion and migration [71, 72]. CCL18 released by breast TAMs promoted the invasiveness of cancer cells by triggering integrin clustering and enhancing their adherence to the extracellular matrix [73]. Another study showed that macrophage migration inhibitory factor (MIF) also promoted tumor metastasis by increasing the prevalence of a highly immunosuppressive subpopulation of MDSCs within the tumor [74].

There are few canine mammary tumor studies regarding the influence of immune cells in tumor invasion and metastasis [56, 75, 76]. The role of lymphocytes and macrophages in

canine mammary tumor metastasis is not fully understood. When a high number of $CD8^+$ T-cells was found in metastatic canine mammary tumors the authors suggested the involvement of this lymphocyte subtype in tumor metastasis [77]. Contrary to this study, another group showed that the relative percentage of $CD4^+$ T-cells was higher in canine mammary tumors that metastasized, whereas $CD8^+$ T-cells were higher in tumors that did not metastasize [78, 79]. In terms of TAMs, their density was significantly higher in canine mammary adenocarcinomas that metastasized [75]. Furthermore, a microarray analysis to determine the global gene expression of a co-culture of canine macrophages and canine mammary cancer cells showed an up-regulation of genes involved in angiogenesis in TAMs and an increase in the migratory and invasive capabilities of cancer cells [76]. Mucha et al. showed, for the first time, that MDSCs demonstrated increased activation of IL-28/IL-28RA (IFN-γ) signaling, which stimulates STAT3 in canine mammary tumor cells therefore promoting epithelial-mesenchymal transition (EMT) and increased invasion and migration [56].

Inflammatory Cells and Cancer Cell Biomarkers

Inflammatory infiltrates and tumor COX-2 expression

Cyclooxygenase (COX) is the enzyme responsible for the biosynthesis of various prostanoids (lipid mediators that have several biological functions) by the conversion of arachidonic acid released from the phospholipid membrane through the action of cytosolic phospholipase A2 [80].

Human and laboratory animal studies report COX-2 upregulation in mammary cancer [81-84] and several lines of evidence now strongly support that this enzyme, during mammary tumorigenesis, mediates tumor survival by several mechanisms: i) inhibiting the tumor cells apoptosis and inducing tumor cells proliferation; ii) increasing tumor progression by altering cells morphology; iii) increasing cells motility and migration; iv) sustaining proliferative signaling; v) inducing the production of metastasis-promoting MMPs and vi) stimulating the tumor angiogenic switch [85, 86].

The COX-2-derived products, mostly prostaglandin (PG) E2 (thought to be the main tumorigenic COX-2-derived product), are known to act not only in classical cancer signaling pathways to promote carcinogenesis in primary tumor cells, but also in the tumor microenvironment which contains multiple resident and infiltrating cells (including immune cells) as well as the growth factors and cytokines released by them [29, 87].

Recent findings revealed that COX-2-produced prostaglandins are potent lipid molecules that act as immunomodulators in key aspects of mammary tumor immunity [88-91].

In human breast cancer COX-2-derived PGE2 has the ability to influence local immune responses in the tumor stroma contributing to tumor evasion of immune surveillance and supporting tumor development and metastasis [85, 91]. PGE2 has diverse effects on the regulation and activity of T-cells [91, 92]. Recently, PGE2 has been implicated in the enhancement of pro-tumorigenic Th2-type cytokine, such as IL-4, IL-5 and IL-10, and inhibition of anti-tumor Th1 cytokine production, such as IFN-γ and IL-2 [87, 89]. The cellular effects of PGE2 are mediated through four receptors, EP1, EP2, EP3 and EP4 associated to different intracellular signaling pathways [93]. The inhibition of the Th1 cell proliferation is dependent of EP2 [94]. The EP2 and perhaps the EP4 receptors mediate the suppressive effects of PGE2 on T-cells [95]. The induction of the Th2 response by PGE2 is modulated most probably by the second messenger cyclic adenosine monophosphate (cAMP), since the biological products that increase the level of cAMP mimic the effects of PGE2 [96, 97]. Even though there is limited knowledge regarding the effects of PGE2 on $CD8^+$ cytotoxic T-cells, it has been shown that, as for Th1 cells, PGE2 can inhibit $CD8^+$ T-cell proliferation, suppress cytotoxic $CD8^+$ T-cell actions against the tumor and, in terms of regulating cytokine production, decrease the production of IFN-γ by $CD8^+$ T-cell clones through a cAMP-dependent pathway [98]. Additionally, the PGE2 effects during the priming of DCs with tumor antigens inhibit completely the DCs ability to produce IL-12 and prompt the production of high levels of IL-10 [99]. In this process the PGE2-primed DCs induce the direct differentiation of naive T-cells into Th2 cells, which further supports the role of COX-2- derived PGE2 in biasing the immune system towards Th2 and away from potentially beneficial Th1 responses in tumor sites [99, 100].

COX-2 has some effects on FoxP3 expression and Treg cell functions. Several studies have demonstrated that Treg cells contribute to immunosuppression in cancer and inhibit effector T-cells in a COX-2-dependent manner in mouse models or in human peripheral blood [101-104]. An intratumoral increase in COX-2 and PGE2 levels is strongly correlated with the up-regulation of FoxP3 and the suppressive capabilities of Tregs in several human cancers [105, 106]. In human breast cancer, COX-2-derived PGE2, acting through EP2 and EP4, increases Treg infiltration, differentiation, and function, which in turn suppress the maturation of other T-cells leading to immunosuppression and increased tumor cell survival [93].

Evidences from clinical and experimental studies indicate that macrophages are versatile cells that are capable of displaying different functional activities in order to promote breast cancer progression and metastasis [19, 107, 108]. TAMs polarization in mammary tumor sites are modulated by the tumor microenvironment and these cells are "educated" adopting a role that facilitates angiogenesis, matrix breakdown and tumor-cell motility in a COX-2-dependent manner [90, 109].

A substantial body of work indicates the immune suppressive role of COX-2 and describes the COX-2/PGE2 modulation of macrophages, including the downregulation of M1 macrophage markers/cytokines, which elicit Th1 immune responses, and the upregulation of M2 macrophage markers/cytokines, which block Th1 immune responses [109, 110]. Moreover, in the murine breast cancer model, the selective COX-2 inhibitors can change the tumor associated macrophage phenotype from M2 to M1 [90].

Considering COX-2/PGE2-mediated immunomodulation in human breast cancer, it is worthy to note that the immunosuppressive cell subtypes, modulated by COX-2 pathways, share common cytokine mediators and, more importantly, each cell subtype can also generate PGE2 providing an autocrine mechanism for prolonging and enhancing their own immunosuppressive phenotype [111]. This emphasizes the importance of exploring the tumor microenvironment as a whole, rather than focusing on alterations in an individual subset of tumor-associated cells [29]. Collectively, these findings show that COX-2 and immune cell share common signaling pathways in mammary carcinogenesis, which are associated with changes in immune cells profiles and functionality and the role of COX-2 may allow neoplastic cells to evade attack by the immune system.

In canine mammary tumors there are only a limited number of studies focusing on the crosstalk between COX-2, cancer cells and immune cells [20, 112] and this topic is incompletely understood. Our team demonstrated a significant association of high COX-2 immunoexpression with $CD3^+$ T-lymphocytes [20] and MAC387 macrophages [112]. Tumors with concurrent high COX-2/CD3 and high COX-2/MAC were statistically associated with variables of tumor aggressiveness (high histological grade of malignancy, presence of neoplastic intravascular emboli and presence of lymph node metastasis) and shorter overall survival of animals [112]. These findings suggest that, similarly to human breast cancer, T-lymphocytes, macrophages and COX-2 share functions in canine mammary carcinogenesis.

Inflammatory infiltrates and receptor tyrosine kinases (RTKs)

Receptor tyrosine kinases (RTKs) are cellular proteins that have been intensively studied [52, 113-115]. Their role in the control of cellular growth and differentiation is central to all organisms and has been found to participate in human and animal neoplastic diseases [116-118].

There are several mechanisms by which tyrosine kinases might acquire transforming functions. RTKs are essential components of cellular signaling pathways that are active during embryonic development and adult homeostasis [119]. Due to their roles as growth factor receptors, many RTKs have been implicated in the onset or progression of various cancers, including mammary cancer, either through receptor gain-of function mutations in the corresponding genes or through receptor/ligand overexpression by autocrine-paracrine growth factor loops [120, 121].

The RTKs family include, among others, the human epidermal growth factor receptor (EGFR) family with its members HER-1/EGFR and HER-2, -3, -4; platelet-derived growth factor receptors (PDGFR, which include c-kit); fibroblast growth factor receptors (FGFRs); VEGF; hepatocyte growth factor/scatter factor receptor (HGF/SF); ephrin receptors (Ephs); and the insulin receptor [120, 122, 123].

Here, we will only focus on the most important RTK in mammary carcinogenesis, the EGFR and c-kit which are often overexpressed or mutated in this type of tumor [52, 118, 124, 125], since VEGF is already described above.

In human breast cancer, RTKs signaling has been described as being implicated in differentiation and migration of immune cells into tumor sites contributing to the immune balance from activation to tolerance which is implicated in tumor progression [114, 126-128]. Consequently, a number of tyrosine kinase inhibitors (TKI) have been developed which, besides direct anti-cancer effects, also increase the number and function of effector immune elements, while decreasing the amount and function of suppressor immune cells [129, 130].

Studies in pre-clinical breast cancer mouse models demonstrated that trastuzumab (monoclonal antibody against HER2) and cetuximab (monoclonal antibody to EGFR/HER-1) both have an important role in tumoral innate and adaptive immunity, inducing natural killer (NK) cells and cytotoxic as well as Th1 T-lymphocytes activity [131-134].

The great homology in the EGFR family among humans and dogs [118] prompted us recently to produce a recombinant canine anti-EGFR antibody for canine mammary tumor treatment [117].

Several lines of evidence suggest that ligands to EGFR family molecules (except HER-2) can be produced by non-tumor cell types. The infiltrating T-lymphocytes in human breast tumors can produce EGF [135] and analysis of breast tumor explants revealed that EGF is also produced by TAMs [113, 114, 136]. In fact, the important role of macrophage-secreted EGFR ligands in malignant mammary tumor progression leads to increased carcinoma cell invasion and metastasis [114].

Interestingly, one study showed that even $FoxP3^+$ Treg cells express EGFR under inflammatory conditions. The stimulation with the EGF-like growth factor amphiregulin markedly contribute to the enhancement of Treg cell functions *in vitro*, and in a tumor vaccination model [137].

In canine mammary tumors there is only one study suggesting that concurrent COX-2/EGFR positive immunoexpression is associated with higher number of intratumoral $CD3^+$ T-lymphocytes. In this work, tumoral $CD3^+$ T-lymphocytes may be influenced by inappropriate expression of COX-2/EGFR. COX-2 over-regulation and the resulting increase in PGE2 levels induced over-expression of EGFR pathways and may represent a strategy adopted by tumors that contributes to the evasion of tumor-specific immune response [20].

In several human tumors, including breast cancer, the stem cell factor (SCF) that triggers the c-kit signaling pathways has been described as possibly being involved in the complex relationship between immune cells and tumor cells in the tumor microenvironment [126, 127]. c-kit upregulation on DCs, induce the activation of several signaling pathways that block IL-12 and promote IL-6 production. c-kit dependent signaling supports an immune twisting toward Th2 and Th17 subsets and away from Th1 responses. The cytokines produced induce T-cells tolerance and contribute for cancer development [126, 138, 139]. Sunitinib and sorafenib, multi-kinase inhibitors that block, among others, the VEGF and c-kit receptors [130], have the ability to modify the mammary tumor microenvironment in multiple ways, including the alteration of immune cell infiltration by immune subset conditioning [140]. These TKI may contribute to the enhancement of Th1 and $CD8^+$ T cells intratumoral infiltration with cytolytic activity against the tumor [130, 141]. Sunitinib could also block the conversion of conventional $CD4^+FoxP3^-$ T-cells into $CD4^+FoxP3^+$ Treg cells [130]. Studies *in vitro* and using tumor-bearing mice showed that

c-kit inhibitors may induce a reduction of Treg cell number, decreasing consequently the expression of immunosuppressive cytokines (IL-10, TGFβ) [142]. Furthermore, in mice, sunitinib prompted anti-angiogenic effects and promoted direct pro-apoptotic properties, resulting in a decline of mammary tumor progression. Regarding TAMs, their density was slightly reduced under sunitinib treatment [143].

Canine mammary tumor studies on the interplay between c-kit and tumor immunology are scarce. However, one study demonstrated a positive correlation between $CD3^+$ T-lymphocytes and c-kit immunoexpression. Tumors with high CD3/c-kit were associated with tumor aggressiveness (high histological grade of malignancy, presence of neoplastic intravascular emboli and presence of lymph node metastasis) and shorter overall survival of animals. The results of this work are a first attempt to explore the possible common signaling pathways between c-kit and immune system in canine mammary carcinogenesis [52].

Therefore, the findings described above prove that RTK pathways are important not only for the remodeling of mammary tumor microenvironment but also could be a very important target for tumor immunological therapy [128].

Concluding remarks

Cancer can take advantage of the stromal inflammation [144]. T-lymphocytes, macrophages and other inflammatory cells in human and the dog mammary tumor microenvironment acquired pro-tumorigenic properties that are crucial to fuel the major biological processes involved in tumor development, progression and metastasis [14, 46, 144, 145].

The involvement of inflammatory cells to tumor hallmark acquisitions by supplying pro-angiogenic growth factors, cytokines and proteases and the recent success of checkpoint inhibitors in clinical oncology approve the great relevance of the dark side of immune regulation for driving cancer.

The similarities described above between humans and dogs prove the value of dog as an important translational model for comparative oncology in the study of the molecular signalling based on tumor immunosuppression. In the future continuing research on this topic seems to be relevant in order to find novel immunotherapies that may target tumor microenvironment interconnected pathways.

Table 7 - Relationship between T-lymphocytic and macrophages infiltrate and tissue biomarkers in human and dog mammary tumors.

Tissue biomarkers	T-lymphocytic and macrophages infiltrate	
	Human breast cancer	**Canine mammary tumors**
Angiogenesis	- T-cells can secret VEGF [39]; - Treg cells release TGFβ1 and induce VEGF expression [40]; - M2 macrophages are responsible for the production of VEGF, uPA, MMP9 and CCL18 promoting tumor neovascularization [48, 51].	- Positive correlation between $CD3^+$ T-cells, VEGF and microvessel density [52]; - M2 macrophages infiltration is associated with VEGF expression [57].
Invasion and metastasis	- IL-10 produced by Th2-polarized $CD4^+$ T lymphocytes promotes M2-TAMs polarization, enhancing metastasis through EGFR signaling activation [64]; - IL-1β elicits IL-17 expression from T-cells, resulting in expansion and polarization of neutrophils that have the ability to suppress cytotoxic $CD8^+$ T-lymphocytes and favors metastatic spread [65]; - MMP2, MMP9 and CCL18 produced by macrophages increase tumor cells motility and invasiveness [68, 73].	- $CD4^+$ T-cells and TAMs density are high in metastatic CMT; - $CD8^+$ T-cells is higher in tumors that not present metastatic behavior [75, 78, 79].
COX-2	- COX-2-derived PGE2 enhance the production of IL-4, IL-5 and IL-10 by Th2 cells and inhibits the anti-tumor Th1 cytokines (IFN-γ and IL-2) [87, 89]; - PGE2 inhibits $CD8^+$ T-cells proliferation and anti-tumor activities [98]; - COX-2/PGE2 pathways increase tumor Treg cells infiltration, differentiation and function [93] and can change the tumor associated macrophages phenotype from M1 to M2 [109, 110].	- Tumors with high COX-2/CD3 and high COX-2/MAC are associated with tumor aggressiveness and shorter OS [112].

Table 7 - Continued

Tissue biomarkers	T-lymphocytic and macrophages infiltrate	
	Human breast cancer	**Canine mammary tumors**
Receptor Tyrosine kinases	- T-lymphocytes and TAMs produce EGFR ligands being involved in tumor progression [113, 114, 135]; - FoxP3$^+$ Treg cells express EGFR under inflammatory conditions, which is related with tumor cells invasion and metastasis [137]; - c-kit dependent signaling supports an immune twisting toward Th2 and Th17 subsets and the cytokines produced induce T-cell tolerance, contributing for cancer development [126, 138, 139]; - c-kit inhibitors induce a reduction of TAMs and Treg cell numbers and contribute to the enhancement of Th1 and CD8$^+$ T-cells[130, 141-143].	- Concurrent COX-2/EGFR expression is associated with higher numbers of tumoral CD3$^+$ T-lymphocytes and characteristics of tumor aggressiveness [20]; - High CD3/c-kit tumors are associated with variables of tumor aggressiveness and shorter overall survival of animals [52].

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Chapter II – The Role of FoxP3 Regulatory T-Lymphocytes, TGFβ and IL-35 in Canine Mammary Carcinogenesis

Original Research Articles:

Intratumoral FoxP3 expression is associated with Angiogenesis and Prognosis in Malignant Canine Mammary Tumors.

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Crosstalk between TGFβ, FoxP3 expression and Angiogenesis in Malignant Canine mammary tumors: association with Clinicopathological Parameters and Prognosis.

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Submitted manuscript

Assessing the Interleukin 35 immunoexpression in Malignant Canine Mammary Tumors: association with Clinicopathological Parameters and Prognosis.

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Submitted manuscript

II.1 Intratumoral FoxP3 expression is associated with Angiogenesis and Prognosis in Malignant Canine Mammary Tumors

Abstract

The activity of regulatory T-cells (Tregs) is closely associated with the expression of FoxP3 transcription factor. FoxP3 regulatory T-cells (FoxP3Treg) have immunosuppressive properties and can work for prevention of harmful autoimmune responses, however can also interfere with beneficial anti-tumor immunity. In human breast cancer these cells play a crucial role in tumor progression. In canine mammary tumors (CMT) this topic is not well-documented. This study included 80 malignant CMT and studied, by immunohistochemistry, the intratumoral FoxP3 expression together with microvessel density (MVD), vascular endothelial growth factor (VEGF) and several clinicopathological characteristics. Abundant FoxP3Treg cells were associated with tumor necrosis ($p = 0.001$), high mitotic grade ($p < 0.001$), more marked nuclear pleomorphism ($p = 0.001$), poor differentiation of tumors ($p < 0.001$), high histological grade of malignancy (HGM) ($p < 0.001$), presence of neoplastic intravascular emboli ($p < 0.001$) and presence of lymph node metastasis ($p < 0.001$). Intratumoral FoxP3 was correlated with MVD ($r = 0.827$; $p < 0.001$) and associated with VEGF ($p = 0.001$). Additionally tumors with abundant FoxP3Treg cells were associated with shorter overall survival (OS) time in univariate and multivariate analysis ($p < 0.001$ Kaplan-Meier curves and 7.97 hazard ratio, $p < 0.001$ Cox proportional hazard model). Results suggest that Treg cells play a role in CMT progression and may contribute to increased angiogenesis and aggression in these tumors. The association of intratumoral FoxP3 expression with shorter OS in multivariate analysis suggests the usefulness of Treg cells as an independent prognostic marker.

Introduction

The activity of regulatory T-cells (Tregs) is known to be closely associated with the expression of FoxP3 transcription factor [1, 2]. FoxP3 regulatory T-cells (FoxP3Treg) are a distinct group of T-lymphocytes that play an important role in the homeostasis of the immune system and in the modulation of the immune response. Tregs have emerged as key players in the development and maintenance of peripheral immune tolerance [3, 4]. Normally these cells can work for prevention of harmful autoimmune responses, however can also interfere with beneficial immune responses such as anti-tumor immunity [1, 3, 5]. Tregs can secrete inhibitory cytokines, such as IL-10, TGFβ which induce the reduction of both $CD8^+$ T-lymphocytes and natural killer (NK) cells cytotoxic activities, thus contributing for anti-tumor immune suppression [6-8]. Recent studies have shown that Tregs are implicated in inducing tumor progression [5, 9-12]. Tumor antigens are recognized by autologous T-cells [13] and are regarded as self by Tregs that actively promote their tolerance [14].

In human studies increased numbers of Treg cells have been associated with tumor aggressiveness and poor survival in many solid tumors, [15, 16] including breast cancer [17-19]. However, in breast tumors some groups have showed the association of Treg cells with good clinical outcome, [20] highlighting the complexity of Tregs as a biomarker.

In dogs augmented Treg frequencies have been linked to tumor stage, prognosis and survival in several tumor types [21-25]. In turn, in classical and spermatocytic canine seminomas Foxp3Treg cells may be associated with a less malignant histological phenotype [26]. In the context of canine mammary tumors (CMT), the importance of Tregs lies in the fact that increased numbers may favor tumor development or growth and influence the course of the disease, [12, 27]. However there are only a few studies and this topic is not well-documented.

Angiogenesis represents a key event in the development of tumors. The absence of oxygen in the intratumoral compartment (hypoxia) induce the expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) resulting in an angiogenic switch that is associated with the proliferation and migration of endothelial cells and the formation of new blood vessels [28-30]. Angiogenesis is regulated by a balance between pro- and anti-angiogenic signals and represents a key event in the development and maintenance of inflammatory disorders such as tumors [28, 29, 31]. The

induction of angiogenesis is mediated by several cells, including Treg cells that produce anti-inflammatory cytokines and VEGF, which is upregulated by hypoxia, playing a central role in the modulation of tumor angiogenic switch [28, 32-34].

In human breast cancer the accumulation of Tregs at tumor sites has been correlated with biomarkers of angiogenesis such as VEGF overexpression and increased microvessel density (MVD) providing clinical indications for an association between Tregs and angiogenesis [35]. Tregs can contribute to tumor angiogenesis through both indirect and direct mechanisms. Tregs promote angiogenesis indirectly by suppressing the activities of Th1 effector T-cells that release angiostatic cytokines as TNF-α and IFN-γ [36]. *In vitro* studies showed that Treg cells secreted higher amounts of VEGF under hypoxic conditions. In hypoxia, Tregs promote also capillary tube formation in a VEGF dependent signaling [31, 32, 37].

In CMT, Treg cells [12, 27] and angiogenic markers (VEGF and MVD) [38, 39] are independently associated with an aggressive tumor phenotype, however the prognostic value and the regulatory role that Treg cells may have on tumor angiogenesis are not well understood yet which justify the need of more studies in this field. The aims of this study were to analyze the intratumoral FoxP3 expression, together with MVD, VEGF, several clinicopathological characteristics and OS of animals, in order to investigate the potential association of Treg cells with angiogenesis and clinical outcome in malignant CMT.

Materials and Methods

Study population and sample collection

A total of 80 malignant mammary tumors, excised surgically from female dogs received for diagnosis and treatment, were included. For this study, one tumor was selected per animal. When more than one malignant neoplasm were diagnosed, the tumor with the more aggressive clinical and histopathological features (larger size, infiltrative growth, higher grade) was selected [40]. The animals ranged in age from 2 to 17 years (mean 10 years), were of different breeds and did not received other treatment such as chemotherapy and/or radiation therapy. The clinical stage of animals were categorized using a modified TNM system [41] in which T describes the size of the primary tumor (higher diameter); N the presence (N1) or absence (N0) of metastasis at regional lymph nodes and M the presence (M1) or absence (M0) of metastasis at distant organs. Following this system 3 clinical stages were established: local (without lymph node

involvement), regional (metastasis at regional lymph nodes) and distant (presence of distant metastasis) [40, 42]. In cases with regional lymph node enlargement, the involvement was investigated by fine needle aspiration and confirmed by histological analysis after surgical removal. On each mammary tumor were also evaluated the tumor size (T1 <3 cm; T2 ≥ 3 and <5 cm; T3 ≥ 5 cm) and the skin ulceration. The absence of distant metastases was investigated throughout the realization of thorax X-ray and abdominal ultrasound.

Histopathological examination

The samples were fixed in 10% neutral buffered formalin and then embedded in paraffin wax. A 4 μm thick section was cut from each tumor sample block and placed on a silane-coated slide glass. The tumor slides were stained with haematoxylin and eosin (HE). Thereafter, each slide was evaluated microscopically, classified using the criteria proposed by the World Health Organization (WHO) for CMT [43] and graded [mitotic index, nuclear grade, differentiation grade, histological grade of malignancy (HGM)] in accordance with the method proposed by Goldschmidt and collaborators [44]. Additional clinicopathological characteristics evaluated were the presence of tumor necrosis, the presence of neoplastic intravascular emboli and the regional lymph node involvement.

Immunohistochemistry

For immunohistochemistry the tumor sections were deparaffinized in xylene and rehydrated in graded alcohol. The FoxP3 staining was performed with a polymeric labeling methodology (Novolink Polymer Detection System; Novocastra, Newcastle, UK), while for CD31 and VEGF was used the streptavidin-biotin-peroxidase complex method with the Ultra Vision Detection System kit (Lab Vision Corporation, Fremont, CA, USA), following the manufacturer's instructions. Antigen retrieval was carried out by boiling tissue section in citrate buffer (pH 6.0) for 15 minutes (3 x 5 minutes cycles) in a microwave (750 W, high power). Slides were cooled and washed three times in phosphate buffered saline (PBS; pH 7.4). The sections (4 μm) were treated with 3% H_2O_2 solution for 20 minutes at room temperature, followed by three washes in PBS. Then they were incubated overnight at 4ºC with primary antibodies: FoxP3 (anti-mouse/human Foxp3 antibody, Clone eBio7979, eBioscience, San Diego, USA; diluted to 1:100); CD31 (Clone JC70A, Dako, Glostrup, Denmark; diluted to 1:20), VEGF (Clone JH121, Thermo Scientific, Waltham, MA USA; diluted to 1:100). The antibody reaction products were

observed with the chromogen 3, 3'-diaminobenzidine tetrachloride (DAB) at 0.05% with 0.01% H_2O_2 (30%). After a final washing in distilled water, the sections were counterstained with Gill's hematoxylin, dehydrated, cleared and mounted. The primary antibody was replaced with an irrelevant isotype-matched antibody for negative control. This study also included adequate positive controls: sections of canine lymph nodes were used as positive control for FoxP3; for CD31 and VEGF was used dog angiosarcoma and liver section respectively.

FoxP3, CD31 and VEGF staining evaluation

To evaluate intratumoral FoxP3 expression, the three regions in the tumor with the most intense positivity were selected. In these regions, all labelled cells were counted, evaluating a total of 10 HPFs (×400) following a quantitative method already adopted by our team for estimate the T-lymphocyte immunostaining [45]. The measurement of microvessel density by CD31 (Platelet Endothelial Cell Adhesion Molecule 1- PECAM 1) and the evaluation of VEGF staining were performed according with previously established methods used in CMT by our group [38, 39, 46]. Respecting the assessment of microvascularization density were identified the two hot spot areas and were counted three different fields in each of these areas. All positive cells or cell clusters were considered as a single countable microvessel and the somatory of the number of microvessels was calculated from the two vascular hot spots [38, 46].

Follow-up study

Dogs were clinically examined by veterinarians before surgery, 15 days after surgery and every 90 days thereafter for a minimum period of 730 days. Each follow-up evaluation included a physical examination, a radiological evaluation of the thorax and an ultrasound scan of liver and kidneys. For the animals that died within the 730 days period, overall survival (OS) was calculated from the date of surgery to the date of animal death/euthanasia due to advanced stages of the disease. For the dogs who survived more than 730 days, the OS was the number of days from surgery to the last clinical examination. Follow-up was carried out in the 80 animals with malignant mammary tumors, for a mean period of 472 days (minimum 37, maximum 730 days).

Statistical analysis

To study categorical variables, chi-square test was performed. Analysis of variance (ANOVA), with Tukey's multiple means comparison, was used for analyzing continuous variables. The Pearson's correlation test was performed in order to verify the presence of correlation between values of FoxP3 and CD31. Survival curves were calculated by the Kaplan-Meier method using the mean values as cut-of and the log-rank test was used for the survival estimates. Multivariate Cox proportional hazards model was also applied. A value of $p < 0.05$ was considered statistically significant. Statistical analysis were done using SPSS software (Statistical Package for the Social Sciences, Chicago, IL, USA) version 19.0.

Results

Clinicopathological data

Mammary tumor samples (n = 80) were examined and classified as: tubulopapillary carcinomas (n = 38), complex carcinomas (n = 14), solid carcinomas (n = 10), anaplastic carcinomas (n = 4) and carcinosarcomas (n = 14). Thirty one tumors had lymph node metastasis. The HGM was classified as I (n = 21), II (n = 19), or III (n = 40).

Expression of FoxP3, CD31 and VEGF in malignant CMT

FoxP3 positive T-lymphocytes showed a predominant diffuse distribution within the intratumoral compartment and were observed in all the cases. FoxP3 immunoreactivity was expressed in the nuclei of the infiltrating T-lymphocytes. The mean number (±SE) of intratumoral FoxP3 cells was 72.66 ± 5.810 (range, 19–267). The CD31 immunoexpression was observed in the membrane of endothelial cells. The mean number (±SE) of total neovessels was 38.51 ± 2.327, minimum 6 and maximum 106.

The VEGF showed a predominant granular cytoplasmic staining pattern more intense in epithelial neoplastic cells. Multifocal staining was observed in stromal cells (<25% of the stromal cells). All tumors stained positively, and the staining varied between weak and strong.

Associations of FoxP3, CD31 and VEGF immunostaining with clinicopathological features

Tumors with higher values of FoxP3 infiltrating T-lymphocytes showed more aggressive phenotypes: tended to have tumor necrosis ($p = 0.001$), higher mitotic index ($p < 0.001$), more marked nuclear pleomorphism ($p = 0.001$), less tubular formation ($p < 0.001$), elevated HGM ($p < 0.001$), presence of neoplastic intravascular emboli ($p < 0.001$) and presence of lymph node metastasis ($p < 0.001$). For MVD counting, associations were observed with larger tumor size ($p < 0.001$), skin ulceration ($p = 0.003$), tumor necrosis ($p = 0.04$), higher mitotic index ($p < 0.001$), nuclear grade ($p < 0.001$), differentiation grade ($p < 0.001$), elevated HGM ($p < 0.001$), presence of neoplastic intravascular emboli ($p < 0.001$) and presence of lymph node metastasis ($p < 0.001$). VEGF positive tumors showed associations with skin ulceration ($p = 0.001$), mitotic index ($p < 0.001$), nuclear grade ($p = 0.001$), differentiation grade ($p = 0.002$), HGM ($p < 0.001$), presence of neoplastic intravascular emboli ($p = 0.004$) and presence of lymph node metastasis ($p = 0.01$). **Tables 1** and **Table 2** highlights all the results described above.

Correlation between FoxP3 and CD31 immunoexpression

A positive correlation between intratumoral FoxP3 infiltrating T-lymphocytes and MVD ($n = 80$; $r = 0.827$; $p < 0.001$) was observed.

Association of intratumoral FoxP3 and MVD with levels of VEGF expression in malignant CMT

The FoxP3 expression in tumors with high VEGF ($n = 59$; mean 83.80 ± 7.202; range 23–267) was higher ($p = 0.001$) than in tumors with low VEGF ($n = 21$; mean 41.38 ± 4.353; range 19–85) (**Figure 1**). Similarly, there was higher MVD ($p < 0.001$) in high VEGF tumors ($n = 59$; mean 46.64 ± 2.342; range 21–106 microvessels), compared to low VEGF tumors ($n = 21$; mean 15.67 ± 1.194; range 6–23 microvessels). The MVD in tumors with high immunoexpression for FoxP3 and VEGF ($n = 30$; mean 54.90 ± 16.31; range: 26–106) was more elevated than the MVD count in tumors with low immunoexpression for both markers ($n = 19$; mean 15.32 ± 5.65; range: 6–23). The mean MVD was also higher in tumors with high immunoexpression of only one of the markers [tumors FoxP3 high/VEGF low ($n = 2$) or tumors FoxP3 low/VEGF high ($n =$

29)] (n = 31; mean 36.87 ± 15.89; range: 19–80), compared to tumors with low expression for both markers (p < 0.001; **Figure 2**).

Table 1 - Relationship between intratumoral FoxP3 T-cells, MVD by CD31 (average number of microvessels) and clinicopathological parameters in malignant CMT.

	Intratumoral FoxP3				**MVD by CD31**			
Clinicopathological parameters	**n**	**Mean**	**SE**	**p**	**n**	**Mean**	**SE**	**p**
Tumor size								
T1 <3cm	28	62.29	8.273		28	30.00 [a]	3.151	
T2 ≥3cm and <5cm	24	77.78	13.424	NS	24	33.17 [a]	3.600	**<0.001**
T3 ≥5 cm	28	79.22	9.616		28	51.56 [b]	4.291	
Skin ulceration								
Absent	56	65.46	7.101	NS	56	33.98	2.500	**0.003**
Present	24	89.46	9.358		24	48.83	4.603	
Histological type								
Tubulopapillary C.	38	68.08	8.153		38	37.26	3.275	
Solid C.	10	92.30	19.719		10	41.70	6.498	
Complex C.	14	52.29	8.043	NS	14	29.50	5.184	NS
Anaplastic C.	4	84.75	30.877		4	56.50	17.304	
Carcinosarcoma	14	88.00	16.122		14	43.50	4.643	
Tumor necrosis								
Absent	35	51.26	5.714	**0.001**	35	31.15	2.938	**0.04**
Present	45	89.00	8.676		45	44.44	3.230	
Mitotic index								
I	22	41.32[a]	3.731		22	23.36[a]	3.266	**<0.001**
II	30	63.57[a]	7.046	**<0.001**	30	37.97[b]	4.158	
III	28	107.04[b]	11.869		28	50.79[c]	2.675	
Nuclear grade								
I	2	23.00 [ab]	0		2	35.00[ab]	6.000	
II	30	49.10 [a]	5.914	**0.001**	30	26.07 [a]	2.638	**<0.001**
III	48	89.46 [b]	8.093		48	46.31 [b]	3.046	
Differentiation grade								
I	10	41.10 [a]	5.442		10	26.50[a]	4.015	
II	25	42.04 [a]	3.913	**<0.001**	25	27.52[a]	3.630	**<0.001**
III	45	96.69 [b]	8.471		45	47,16[b]	2.971	
HGM								
I	21	41.57[a]	4.043	**<0.001**	21	26.71[a]	3.327	**<0,001**
II	19	40.89[a]	3.436		19	22.42[a]	3.166	
III	40	104.08[b]	8.896		40	52.20[b]	2.684	
Neoplastic intravascular emboli								
Absent	56	57.14	5.845	**<0.001**	56	32.02	2.584	**<0,001**
Present	24	108.88	10.695		24	53.42	3.402	
Lymph node metastasis								
Absent	49	51.63	5.583	**<0.001**	49	30.35	2.514	**<0,001**
Present	31	105.90	9.509		31	51.42	3.428	

n, number of samples; SE, standard error; p, statistical significance; C, carcinoma; NS, not significant. Mean values with different superscript letters denote statistically significant differences on each item considered – Tukey Post Hoc Test.

Table 2 - Relationship between VEGF class and clinicopathological parameters in malignant CMT.

Clinicopathological parameters	Low VEGF n	High VEGF n	p
Tumor size			
T1 <3cm	10	18	
T2 ≥3cm and <5cm	8	16	NS
T3 ≥5 cm	5	23	
Skin ulceration			
Absent	18	38	NS
Present	3	21	
Histological type			
Tubulopapillary C.	12	26	
Solid C.	2	8	NS
Complex C.	5	9	
Anaplastic C.	0	4	
Carcinosarcoma	2	12	
Tumor necrosis			
Absent	12	23	NS
Present	9	36	
Mitotic index			
I	14	8	**<0.001**
II	7	23	
III	0	28	
Nuclear grade			
I	0	2	**0.001**
II	15	15	
III	6	42	
Differentiation grade			
I	4	6	**0.002**
II	12	13	
III	5	40	
HGM			
I	11	10	**<0.001**
II	9	10	
III	1	39	
Neoplastic intravascular emboli			
Absent	26	36	**0.004**
Present	1	23	
Lymph node metastasis			
Absent	20	30	**0.01**
Present	1	29	

n, number of samples; p, statistical significance; C, carcinoma; NS, not significant.

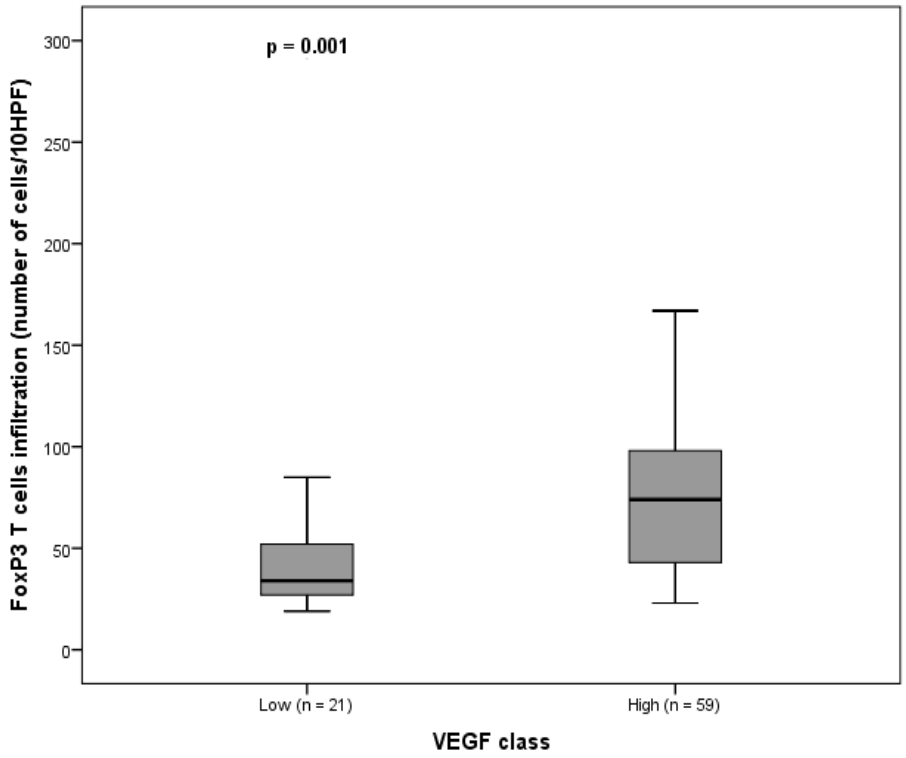

Figure 1 – FoxP3 T-cells distributed according to the VEGF class low or high and respective value of statistical significance for the ANOVA test.

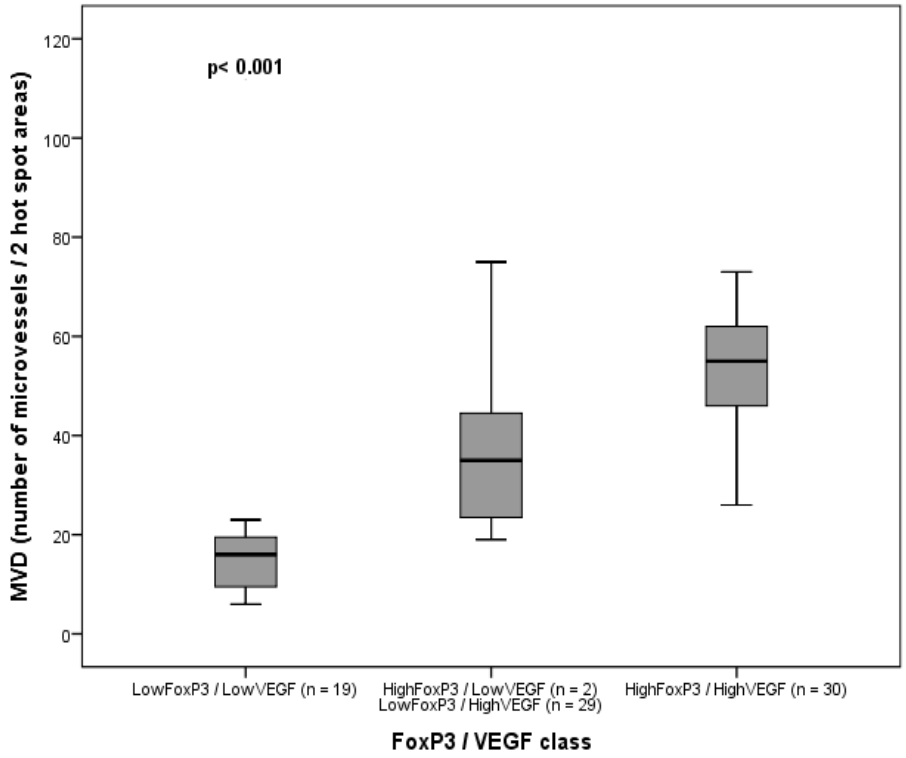

Figure 2 - Association of MVD (number of microvessels) distributed according to the FoxP3/VEGF class and respective value of statistical significance for the ANOVA test.

FoxP3, CD31 and VEGF associations with OS

Tumors with high intratumoral FoxP3 expression ($p < 0.001$), high MVD ($p < 0.001$) and high VEGF expression ($p = 0.003$) were associated with shorter OS time (**Figures 3-5**).

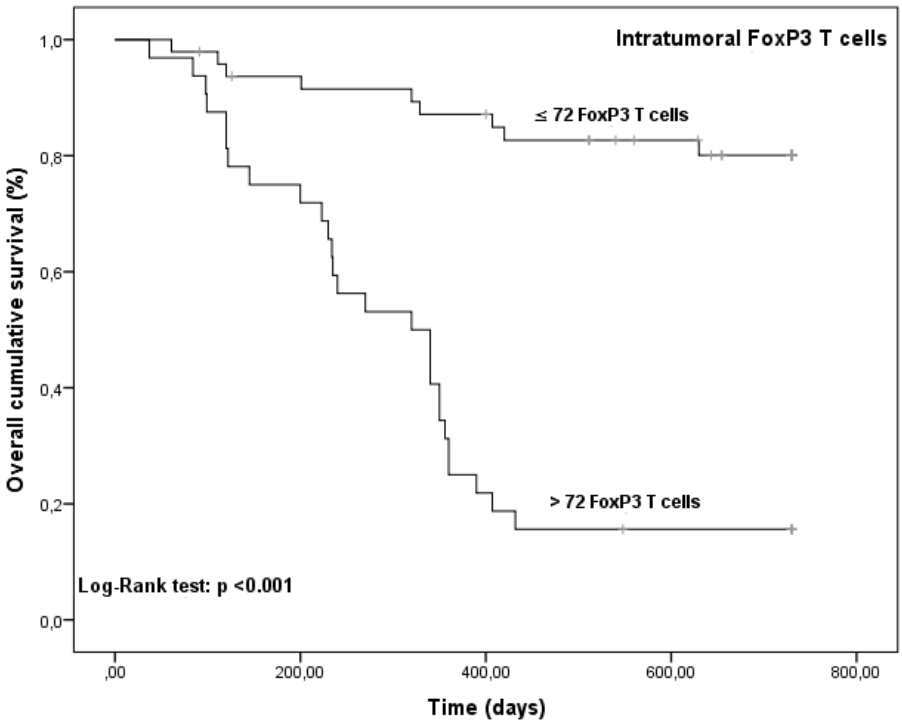

Figure 3 - Kaplan-Meier OS curves comparing FoxP3 T-cells categories in 80 dogs with malignant mammary tumors.

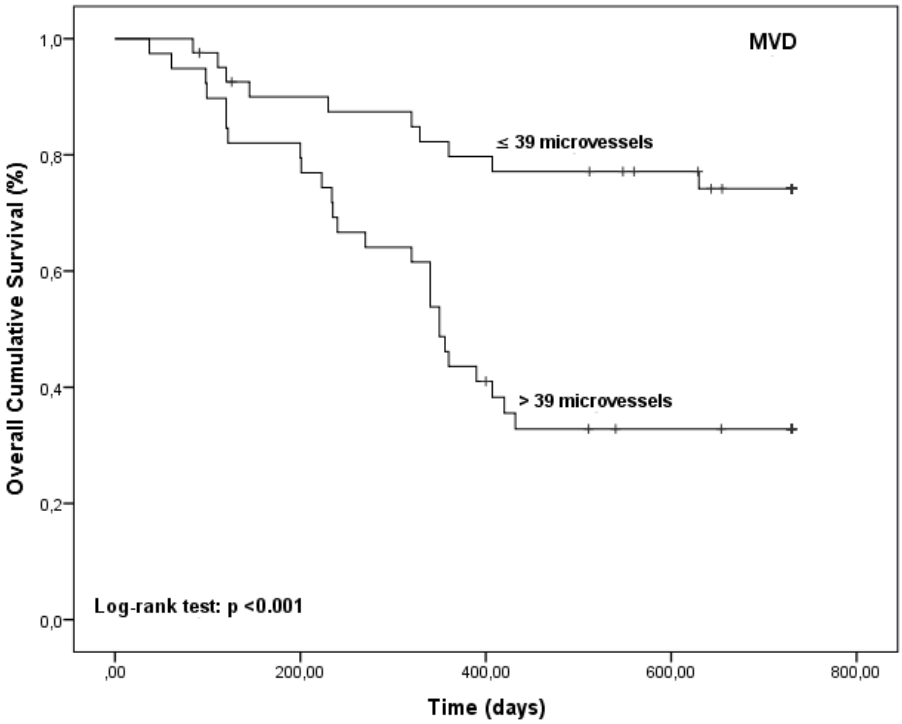

Figure 4 - Kaplan-Meier OS curves comparing MVD categories in 80 dogs with malignant mammary tumors.

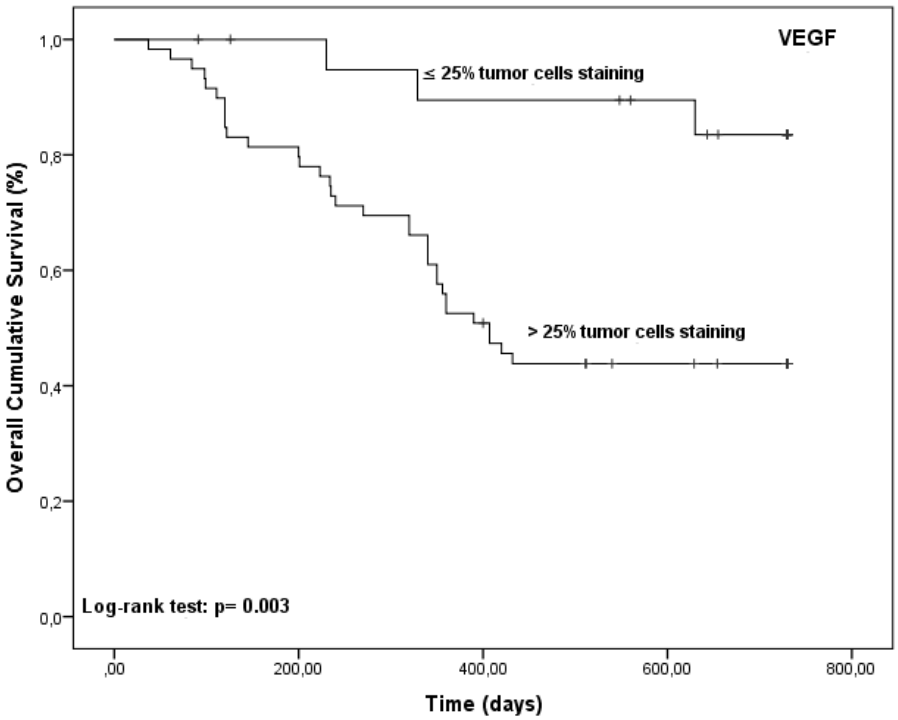

Figure 5 - Kaplan-Meier OS curves comparing VEGF categories in 80 dogs with malignant mammary tumors.

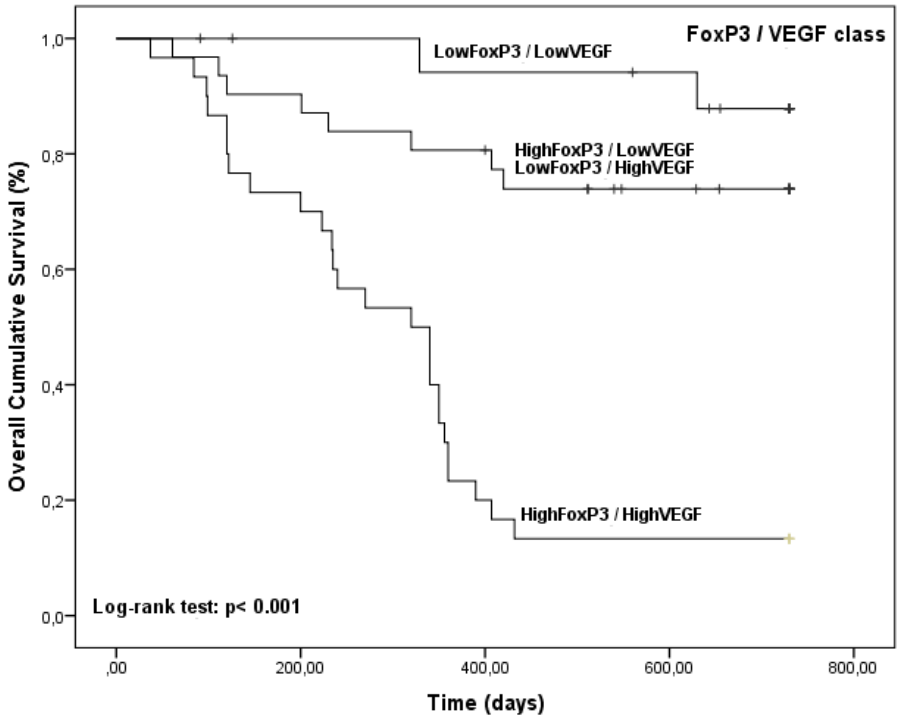

Figure 6 - Kaplan-Meier OS curves comparing FoxP3/VEGF categories in 80 dogs with malignant mammary tumors.

Tumors with concurrent high immunoexpression of FoxP3 and VEGF was associated with poor prognosis ($p < 0.001$; **Figure 6**). Tumors with high intratumoral FoxP3 infiltrating T-lymphocytes retained their significance, by multivariate Cox proportional

hazard model analysis, arising as independent predictor of poor prognosis [Hazard ratio (95% CI): 7.97 (3.68-17.27); p < 0.001]. **Table 3** describes all the results observed in the study.

Table 3 - Association between considered molecular markers and overall survival times.

Molecular markers	n	Overall Survival Univariate (Mean values)	p	Overall Survival Multivariate* (Hazard ratio)	p
Intratumoral FoxP3 T-cells					
Low (≤ 72 FoxP3 T-cells)	48	643.991	**<0.001**	**7.971** (CI: 3.679 - 17.270)	**<0.001**
High (> 72 FoxP3 T-cells)	32	326.625			
MVD					
Low (≤ 39 microvessels)	41	613.969	**<0.001**	-	NS
High (> 39 microvessels)	39	412.639			
VEGF					
Low (≤25% tumor cells staining)	21	676.614	**0.003**	-	NS
High (>25% tumor cells staining)	59	460.148			
FoxP3/VEGF class					
LowFoxP3/LowVEGF	19	700.137	**<0.001**	-	NS
HighFoxP3/LowVEGF or LowFoxP3/HighVEGF	31	601.085			
HighFoxP3/HighVEGF	30	316.400			

n, number of samples; p, statistical significance; NS, not significant; CI, confidence interval; *Multivariate Cox Proportional Hazard Analysis (Cox Regression) of overall survival (OS) in dogs with malignant mammary tumors i prospective study with 2 years of follow-up.

Discussion

Increasing evidences indicate that tumors can influence their cellular milieu to evade immune system [5, 47-49]. The key role of Treg cells in tumor immunity are widely accepted and this topic is under active investigation, both in humans [15-17, 19] and dogs [12, 21, 22, 27]. Large numbers of FoxP3 T-lymphocytes have been found in tumor microenvironment of human breast cancer and increased Treg infiltrations in these tumors

were shown to be associated with worse clinical outcomes [17-19, 50]. Additionally, in human breast cancer, Tregs are present not only in tumor microenvironment of patients but also in tumor-draining lymph nodes and in peripheral blood, suggesting that the increase of Tregs is a generalized phenomenon [19].

In veterinary literature reports suggests a link between increasing numbers of Treg cells and mammary tumors aggressiveness and progression [12, 27]. However, in CMT, this topic is not well-documented, there are only a few studies and some of them include a small number of cases and do not describe histopathological features [24].

In the present study, we demonstrated aggressive phenotypes associated to tumors with higher values of FoxP3. Treg cells infiltrated more frequently tumors with high HGM, presence of neoplastic intravascular emboli and presence of lymph node metastasis. Our results are in agreement with other studies in human [19] and dog mammary tumors [12, 27]. Additionally, present results demonstrated that the higher numbers of Treg cells within the intratumoral compartment were associated with shorter OS of animals, both in univariate and multivariate analysis, which is an innovative finding not described previously. These findings prove the value of FoxP3 Treg cells as an independent prognostic factor in CMT and support the hypothesis that Treg cells allows the tumor cells to evade the antitumor immune responses and may facilitate CMT progression and development. In humans there are studies that revealed a negative role of Treg cells in the prognosis of breast cancer patients [11, 17, 51] and the independent prognostic value of these immune cells was also demonstrated [50]. In CMT, only recently a study demonstrated a significant association of Tregs and poor disease-free survival time by univariate survival analysis [27]. The absence of other studies in CMT field preclude more adequate comparisons of our results. Tregs can secrete inhibitory cytokines, such as IL-10 and TGFβ inducing tumors tolerance [6-8].

There are *in vitro* and *in vivo* studies suggesting that Tregs in tumor sites can use granzyme B to suppress the antitumor activities of NK and $CD8^+$ T-lymphocytes and this suppression is perforin-dependent [52]. Furthermore Tregs, upon in vitro activation, can kill autologous $CD8^+$ cells by Fas/FasL-mediated apoptosis [53]. Our results suggests that in CMT a similar mechanisms should be present explaining the association between Treg cells infiltration, tumor aggressiveness and poor prognosis.

Tumor angiogenesis is mediated by several cells and, in human breast cancer, the accumulation of Tregs at tumor sites has been correlated with the overexpression of VEGF and the increased MVD [35]. In CMT, Treg cells [12] and angiogenic markers

(MVD and VEGF) [38, 39] are independently associated with clinicopathological parameters of tumor aggressive phenotypes, however there are no studies focused on the role of Tregs in tumor angiogenesis. In our study, MVD and VEGF were associated with parameters of tumor malignancy (high HGM, presence of neoplastic intravascular emboli and lymph node metastasis) and poor prognosis (univariate analysis) concordantly with human breast cancer [54-56] and CMT [39]. Interestingly, present study demonstrated a correlation between high intratumoral FoxP3 and high MVD. Additionally tumors with concurrent high FoxP3 and VEGF immunoexpression were associated with poor prognosis. Present results are in agreement with one study in human mammary tumors [35] and support the evidences that activated Tregs release biological mediators, which may induces overexpression of VEGF leading to increased vascularity, tumor aggressiveness and progression. In humans, Tregs are directly involved in promoting angiogenic reprogramming of the mammary tumors microenvironment [31] and the results of present study indicate that, similarly to human breast cancer, in CMT Tregs can make significant contributions to the promotion of tumor aggressiveness and angiogenesis.

Conclusion

Treg cells seem to have an important role in CMT progression and aggressiveness and may share common signaling pathways with VEGF to the direct promotion of tumor angiogenic switch. Thus, we believe that Tregs have a key role in malignant CMT development, linking immune suppression and angiogenesis together in the same biologic program. It is noteworthy that FoxP3 Treg cells retained their significant relationship with OS by multivariate analysis arising as a reliable independent predictor of poor prognosis in malignant CMT. These results open new perspectives in CMT treatment based in specifically targeting Treg cells in order to promote antitumor immunity and tumor regression.

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II.2 Crosstalk between TGFβ, FoxP3 and Angiogenesis in Malignant Canine Mammary Tumors: association with Clinicopathological Parameters and Prognosis

Abstract

Transforming growth factor-β (TGFβ) and FoxP3 regulatory T-cells (Treg) are involved in human mammary carcinogenesis. In canine mammary tumors (CMT) this topic is not well documented yet. This study included 67 malignant CMT and studied, by immunohistochemistry, the tumoral TGFβ levels, the FoxP3, VEGF, CD31 expression and several clinicopathological characteristics. The high levels of TGFβ were associated with skin ulceration ($p = 0.018$), tumor necrosis ($p = 0.024$), high mitotic index ($p < 0.001$), marked nuclear pleomorphism ($p = 0.001$), poor tumor differentiation ($p < 0.001$), high histological grade of malignancy HGM ($p < 0.001$), presence of neoplastic intravascular emboli ($p < 0.001$) and presence of lymph node metastases ($p < 0.001$). The levels of TGFβ were positively correlated with intratumoral FoxP3 ($r = 0.719$; $p < 0.001$), VEGF ($r = 0.378$; $p = 0.002$) and CD31 ($r = 0.511$; $p < 0.001$). Tumors with concurrent high expression of TGFβ/FoxP3, TGFβ/VEGF and TGFβ/CD31 markers were associated with parameters of tumor malignancy (high HGM, presence of neoplastic vascular emboli and presence of lymph node metastasis). Additionally tumors with abundant TGFβ and with concurrent high expression of TGFβ/FoxP3, TGFβ/VEGF and TGFβ/CD31 were associated with shorter overall survival (OS) time ($p < 0.001$). Interestingly TGFβ/FoxP3 class retained the association with shorter OS in multivariate analysis, arising as independent predictor of poor prognosis (9.731 hazard ratio, $p < 0.001$). Results of this study suggest that TGFβ and Treg cells share important pathways contributing to CMT progression, aggression and angiogenesis.

Introduction

Transforming growth factor-β (TGFβ) is a multitasking cytokine expressed in a variety of tissues and exert its activities through 2 serine-threonine kinases receptors: TGFβRI and TGFβRII [1-3]. Once the ligand is activated, TGFβ signaling is mediated through SMAD and non-SMAD pathways. The SMAD signaling pathway requires the phosphorylation and subsequent translocation of SMAD complexes to the nucleus interacting with transcriptional co-regulators and other factors to mediate target gene expression or repression [4, 5]. Although less frequent the non-SMAD pathways contribute to cell proliferation, motility, and survival by activation of the p38 MAPK, p42/p44 MAPK, Rho GTPase, PI3K/Akt signaling [6, 7].

The biologic functions of TGFβ actively participate in key homeostatic cellular pathways, including apoptosis, proliferation and immunity [8]. Furthermore TGFβ is critically important for mammary morphogenesis and secretory function through specific regulation of epithelial proliferation, apoptosis, and extracellular matrix [9]. However there is accumulating evidence that TGFβ signaling are tightly connected and play an important role in malignant transformation in breast cancer, participating in cancer cell migration, survival and angiogenesis [9-12].

TGFβ demonstrates a paradoxical role in malignant mammary tumor process. In early stages of carcinogenesis seems to restrain growth and serves as a tumor suppressor. However, with the development of malignancy, TGFβ becomes a promoter of tumor cell invasion and metastasis [3, 13, 14].

In breast cancer, the dysregulation of TGFβ pathways have been correlated with disease progression, allowing cancer cells to warrant their own survival [12, 14, 15]. In these tumors, TGFβ seems to shape the tumor microenvironment and the TGFβ produced in excess by tumor cells may act in a paracrine manner on the peritumoral stroma, tumor neovessels and immune system resulting in increased cell-matrix interaction, increased angiogenic activity and suppressed immune surveillance which fosters tumor development [10, 16-18].

Mammary cancer cells that have avoided the tumor-suppressive roles of TGFβ can take advantage of its potent immunosuppressive functions. TGFβ signaling in T-cells represses both their inflammatory and cytotoxic differentiation programs [14, 19]. In addition to impairing T-cells effector functions, TGFβ plays a pivotal role in the generation of regulatory T-cells (Tregs) from a population of peripheral $CD4^{+}CD25^{-}$ T-

cells through the induction of the key transcription factor FoxP3 [20, 21]. In human breast cancer the TGFβ and FoxP3 shared signaling pathways have a crucial impact in several tumor hallmark steps, including angiogenesis, facilitating nutrient exchange and metastasis that result in tumor progression [11, 19, 20]. TGFβ and FoxP3 are reported to be sufficient to upregulate the expression of vascular endothelial growth factor (VEGF) which is one of the most selective and potent angiogenic factors known, attracting adjacent endothelial cells and promoting the formation of tumor neovascularization [11, 22].

Recent data in human mammary tumors prove the role of TGFβ and FoxP3 in VEGF signaling and in tumor angiogenic switch with an increased intratumoral microvessel density, contributing to mammary carcinogenesis and poor prognosis [11].

In canine mammary tumors findings suggest that in a cell line TGFβ induces transiently invasiveness capacity [23]. Furthermore Treg cells seems to play a role in CMT development and aggressiveness and may contribute to increased angiogenesis [24]. Another study showed that $FoxP3^{+}CD4^{+}$ T-cells in dogs could be expanded *in vitro* after the addition of TGFβ and IL-2 and by T-cell receptor (TCR) activation [25]. However the prognostic value and the role that TGFβ may have on FoxP3 Treg cells and dog mammary carcinogenesis hasn´t been investigated yet.

The present study used immunohistochemistry to detect TGFβ, intratumoral FoxP3 Treg cells, VEGF, and microvessel density (MVD), and followed up the 2 years overall survival rate, to elucidate the potential association of TGFβ and FoxP3 with angiogenesis and clinical outcome in malignant CMT.

Materials and Methods

Patient selection, sample collection, clinicopathological characteristics, treatment and follow up

A total of 67 dogs received for diagnosis and treatment, were included in this study. The patients were all female with malignant mammary tumors, of different breeds, 2‑17 years old with the mean age of 10.45 years. All female dogs were free from distant metastasis at the time of diagnosis (confirmed throughout the realization of thorax X-ray and abdominal ultrasound), were submitted to surgery for treatment and did not received other parallel treatments such as chemotherapy and/or radiation therapy. One tumor was selected per animal. When more than one malignant neoplasm was diagnosed, the tumor

with the most aggressive clinical and histopathological features (larger size, infiltrative growth, higher grade) was selected [26].The clinical stage of animals was categorized into: local (without lymph node involvement), regional (metastasis at regional lymph nodes) and distant (presence of distant metastases) [26, 27] by the modified TNM system [28] in which T describes the size of the primary tumor (higher diameter); N the presence (N1) or absence (N0) of lymph node metastasis and M the presence (M1) or absence (M0) of metastasis at distant organs. On each mammary tumor was also evaluated the tumor size (T1 <3 cm; T2 ≥ 3 and <5 cm; T3 ≥ 5 cm) and the skin ulceration. For the clinical follow-up, the female dogs included in the study were examined 15 days after surgery and every 90 days thereafter for a maximum period of 730 days. This clinical follow-up examination included a physical checkup, a radiological evaluation of the thorax and an abdominal ultrasound scan. The time of overall survival (OS) was calculated from the date of surgery to the date of animal death/euthanasia due to advanced stages of the disease for the animals that died within the 730 days period or from the date of surgery to the last clinical examination for the dogs that survived more than 730 days.

Histopathological examination

The samples were fixed in 10% neutral buffered formalin and then embedded in paraffin. The paraffin wax blocks were cut into 4 μm thick section and stained with haematoxylin and eosin (HE) following routine methods. Thereafter, each slide was classified using the criteria proposed by the World Health Organization (WHO) for CMT [29] and graded [mitotic index, nuclear grade, differentiation grade, histological grade of malignancy (HGM)] in accordance with the method proposed by Goldschmidt and collaborators [30]. Additional clinicopathological characteristics evaluated were presence of tumor necrosis, presence of neoplastic intravascular emboli and regional lymph node involvement.

Immunohistochemistry

FoxP3 immunostaining was performed with a polymeric labeling methodology (Novolink Polymer Detection System; Novocastra, Newcastle, UK), while for TGFβ, VEGF and CD31 was used the streptavidin-biotin-peroxidase complex method with the Ultra Vision Detection System kit (Lab Vision Corporation, Fremont, CA, USA), following the manufacturer's instructions. Antigen retrieval was performed by microwave treatment for 3x5 min at 750W in 0.01 M citrate buffer, pH = 6.0, followed by cooling at room temperature for 20 min. All sections were then incubated overnight at 4ºC with primary

antibodies: TGFβ (polyclonal antibody against TGFβ1, Santa Cruz Biotechnology, Dallas, Texas, USA; diluted to 1:100) FoxP3 (anti-mouse/human Foxp3 antibody, Clone eBio7979, eBioscience, San Diego, USA; diluted to 1:100), VEGF (Clone JH121, Thermo Scientific, Waltham, MA USA; diluted to 1:100), CD31 (Clone JC70A, Dako, Glostrup, Denmark; diluted to 1:20). The antibody reaction products were observed with the chromogen 3, 3'-diaminobenzidine tetrachloride (DAB) at 0.05% with 0.01% H_2O_2 (30%). After a final washing in distilled water, the sections were counterstained with Gill's hematoxylin, dehydrated, cleared and mounted. The primary antibody was replaced with an irrelevant isotype-matched antibody for negative control. This study also included adequate positive controls: intestine sections were used as positive control for TGFβ; sections of canine lymph nodes were used as positive control for FoxP3; for VEGF and CD31 was used liver section and dog angiosarcoma respectively.

TGFβ, FoxP3, VEGF and CD31 staining evaluation

The evaluation of intratumoral FoxP3, VEGF and microvessel density by CD31 (Platelet Endothelial Cell Adhesion Molecule 1- PECAM 1) was performed according with previously established methods used in CMT by our group [24, 31-33].

The TGFβ immunoreactivity was evaluated by two observers that analyzed the entire slide at 200x magnification power, and used an immunohistochemical semiquantitative method adapted from previous published studies [10]. This method was scored according to the percentage of positive cells (immunolabelling extension) and staining intensity. The percentage of positive cells was scored as 0 (0% positive cells), 1 (<10% positive cells), 2 (10–50% positive cells), 3 (51–80% positive cells), or 4 (>80%). The staining intensity was scored as 0 (no staining), 1 (weakly stained), 2 (moderately stained), and 3 (strongly stained). If the product of multiplication between staining intensity and the percentage of positive cells was ≤ 6, it was consider as low TGFβ class. A final immunohistochemical score > 6 indicates a high TGFβ class.

Statistical analysis

Statistical analysis were done using SPSS software (Statistical Package for the Social Sciences, Chicago, IL, USA) version 19.0. To study categorical variables, a chi-square test was performed. Analysis of variance (ANOVA), with Tukey's multiple means comparison, was used for analyzing continuous variables. The Pearson's correlation test

was performed in order to verify the presence of correlation between values of FoxP3 and CD31. The correlation between non-parametric variables was performed by Spearman's correlation test. Survival curves were obtained with Kaplan-Meier method using the mean values as cut-of and the log-rank test was used for the survival estimates. Multivariate analysis was performed using Cox proportional hazards regression. $p < 0.05$ denotes the presence of a statistically significant difference.

Results

Clinicopathological data

The majority of the tumors included in this study were histologically classified as tubulopapillary carcinomas (n = 38). Others include 8 solid carcinomas, 12 complex carcinomas, 3 anaplastic carcinomas and 13 carcinosarcomas. Twenty eight tumors had lymph node metastasis. Twenty one tumors presented intravascular neoplastic emboli. The HGM was classified as I (n = 17), II (n = 18), or III (n = 32).

Expression of TGFβ, FoxP3, VEGF and CD31 in malignant CMT

Part of FoxP3, VEGF and CD31 cases included in this work were already used in a study published recently by our team [24], and their staining pattern was already described [24]. The mean number (±SE) of intratumoral FoxP3 cells was 73.88 ± 6.585 (range, 19–267; **Figure 7A**). The mean number (±SE) of total neovessels was 39.01 ± 2.562 (range, 6–106).

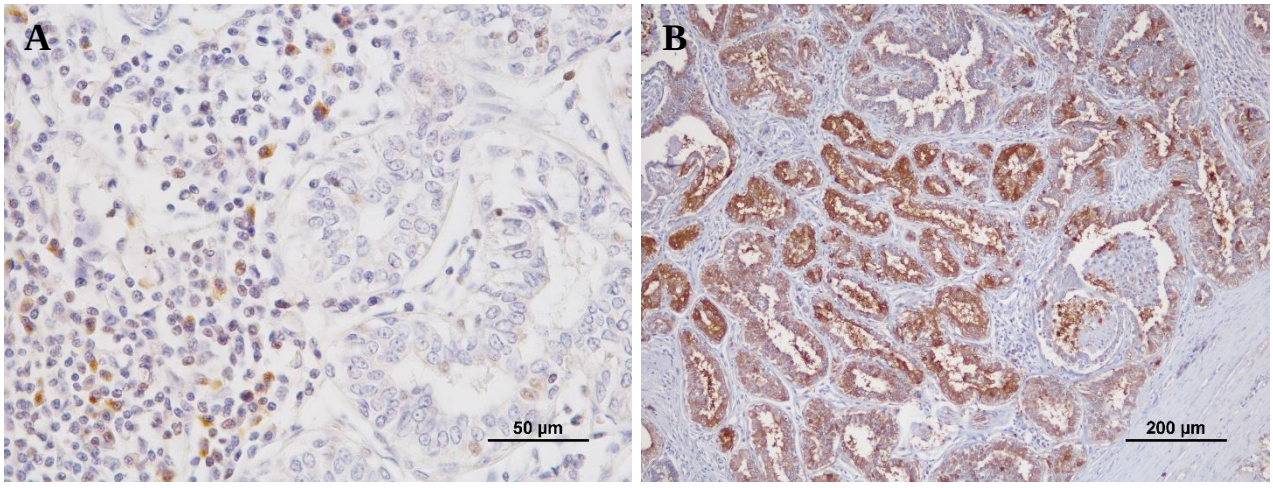

Figure 7 – (A) FoxP3^{+} expression in the nuclei of lymphoid cells infiltrating a tubulopapillary carcinoma, bar = 50 µm; **(B)** Tubulopapillary carcinoma with high expression of TGFβ, note the most evident diffuse cytoplasmic staining, with prominence of the cytoplasmic membrane, bar = 100 µm.

The anti-TGFβ antibody demonstrated high affinity for tumor epithelial cells. The TGFβ immunoexpression predominantly appeared as diffuse or granular cytoplasmic staining, most evident in the cytoplasm of the ductal epithelium, with prominence of the cytoplasmic membrane (**Figure 7B**). Regarding TGFβ immunolabelling extension, briefly 10 cases showed extension 1 (<10% positive cells), 18 cases showed extension 2 (10–50% positive cells), 23 cases and 16 cases demonstrated extension 3 (51–80% positive cells) and 4 (>80%) respectively. For TGFβ labelling intensity, there was also a relatively homogeneous distribution between moderate (40.3%, n = 27) and strong labelling (35.8%, n = 24), whereas tumors with weak intensity (23.9%, n = 16) were less frequent.

Associations of TGFβ immunostaining with clinicopathological features

Our analysis has identified a striking association between the presence of aggressive disease and high expression of TGFβ. Tumors with higher levels of TGFβ were associated with skin ulceration ($p = 0.018$), tumor necrosis ($p = 0.024$), high mitotic index ($p < 0.001$), marked nuclear pleomorphism ($p = 0.001$), poor tumor differentiation ($p < 0.001$), high HGM ($p < 0.001$), presence of neoplastic intravascular emboli ($p < 0.001$) and presence of lymph node metastasis ($p < 0.001$). **Table 4** highlight all the results described above.

Correlation between TGFβ, FoxP3, VEGF and CD31 immunoexpression

The levels of TGFβ were positively correlated with intratumoral FoxP3 ($r = 0.719$; $p < 0.001$), VEGF ($r = 0.378$; $p = 0.002$) and CD31 ($r = 0.511$; $p < 0.001$). In this study three classes were considered: TGFβ/FoxP3, TGFβ/VEGF and TGFβ/CD31. Each class was divided in three categories: 1) low immunoreactivity for both markers; 2) low immunoreactivity for one marker and high for other and 3) high immunoreactivity for both markers.

Association of TGFβ/VEGF class with intratumoral FoxP3 and MVD in malignant CMT

The FoxP3 expression in tumors with concurrent high TGFβ/VEGF immunoexpression (n = 23; mean 118.26 ± 12.535; range: 32–267) was higher than in tumors with low immunoexpression for both markers (n = 15; mean 41.80 ± 5.446; range: 19–85). The FoxP3 expression was also higher in tumors with high immunoexpression of only one

of the markers [tumors TGFβ low/VEGF high (n = 28) or tumors TGFβ high/VEGF low (n = 1)] (n = 29; mean 55.28 ± 6.586; range: 23–186), compared to tumors with low expression for both markers (p < 0.001; **Figure 8**).

Table 4 - Relationship between TGFβ class and clinicopathological parameters in malignant canine mammary tumors.

Clinicopathological parameters	Low TGFβ n	High TGFβ n	p
Tumor size			
T1 <3cm	19	6	NS
T2 ≥3cm and <5cm	12	7	
T3 ≥5 cm	11	10	
Skin ulceration			
Absent	35	13	**0.018**
Present	8	11	
Histological type			
Tubulopapillary C.	22	9	NS
Solid C.	4	4	
Complex C.	9	3	
Anaplastic C.	0	3	
Carcinosarcoma	5	8	
Tumor necrosis			
Absent	23	6	**0.024**
Present	20	18	
Mitotic index			
I	16	1	**<0.001**
II	20	7	
III	7	16	
Nuclear grade			
I	2	0	**0.001**
II	23	3	
III	18	21	
Differentiation grade			
I	9	0	**<0.001**
II	19	3	
III	15	21	
HGM			
I	16	1	**<0.001**
II	17	1	
III	10	22	
Neoplastic intravascular emboli			
Absent	36	10	**<0.001**
Present	7	14	
Lymph node metastasis			
Absent	34	5	**<0.001**
Present	9	19	

n, number of samples; p, statistical significance; C, carcinoma; NS, not significant.

Similarly results were observed for MVD. Tumors with high TGFβ/VEGF immunoexpression (n = 23; mean 53.70 ± 2.872; range: 22–89) showed higher values of microvessels compared with tumors with low immunoexpression for both markers (n = 15; mean 15.40 ± 1.337; range: 6–21). The mean MVD was also higher in tumors with high immunoexpression of only one of the markers [tumors TGFβ low/VEGF high (n = 28) or tumors TGFβ high/VEGF low (n = 1)] (n = 29; mean 39.59 ± 3.705; range: 9–106), compared to tumors with low expression for both markers (p < 0.001; **Figure 9**).

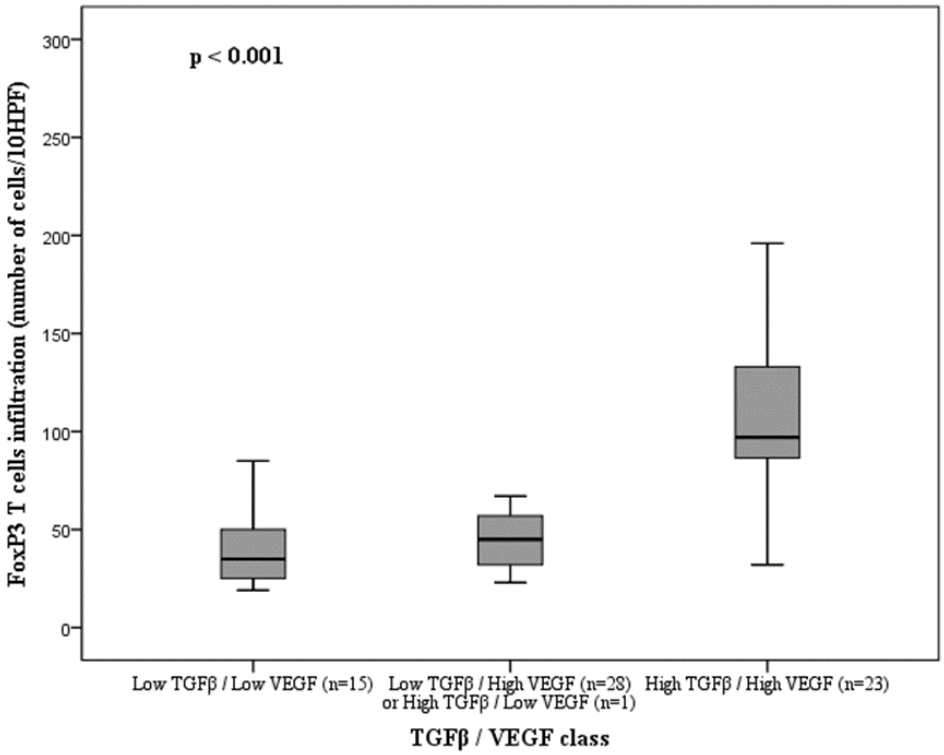

Figure 8 - FoxP3 T-cells distributed according to the TGFβ/VEGF class and respective value of statistical significance for the ANOVA test.

Relationship of TGFβ/FoxP3, TGFβ/VEGF and TGFβ/CD31 classes with clinicopathological variables of tumor aggressiveness

Tumors with concurrent high expression of TGFβ/FoxP3, TGFβ/VEGF and TGFβ/CD31 markers were associated with parameters of tumor malignancy: high HGM (p < 0.001 for TGFβ/FoxP3, TGFβ/VEGF and TGFβ/CD31), presence of neoplastic intravascular emboli (p < 0.001 for TGFβ/FoxP3 and TGFβ/CD31; p = 0.001 for TGFβ/VEGF) and presence of lymph node metastasis (p < 0.001 for TGFβ/FoxP3, TGFβ/VEGF and TGFβ/CD31). More information is provided in **Table 5**.

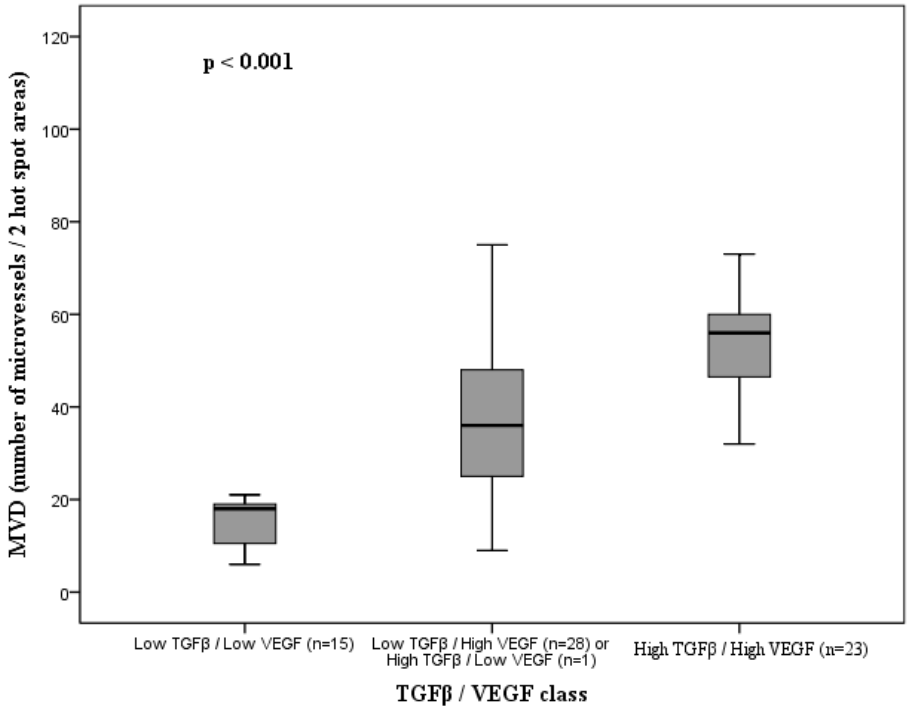

Figure 9 - Association of MVD (number of microvessels) distributed according to the TGFβ/VEGF class and respective value of statistical significance for the ANOVA test.

Follow-up study

Tumors with abundant TGFβ levels and with concurrent high expression of TGFβ/FoxP3, TGFβ/VEGF and TGFβ/CD31 were associated with shorter OS time ($p < 0.001$ for TGFβ/FoxP3, TGFβ/VEGF and TGFβ/CD31 in Kaplan-Meier curves; **Figures 10-13**). Tumors with high TGFβ/FoxP3 expression retained the association with shorter OS in multivariate Cox proportional hazard model analysis, arising as an independent predictor of poor prognosis [Hazard ratio (95% CI): 9.731 (3.982-23.780); $p < 0.001$]. **Table 6** summarizes all the results described above.

Discussion

TGFβ demonstrates a dual role in malignant tumor development process. During the early stages of carcinogenesis, TGFβ acts as a tumor suppressor, negatively regulating cellular proliferation. However, with the development of malignant tumor, the TGFβ role is changed toward a tumor promoter function mediating tumor cells proliferation, migration and invasion. [9, 10, 14]

Findings suggest that the dysregulation of TGFβ pathways in tumors induce signal reprogramming, allowing cancer cells to mimic normal functions to guarantee their subsistence. In fact recent studies have demonstrated that high expression levels of TGFβ

have a close association with several human malignancies [34, 35], including breast cancer [16, 17].

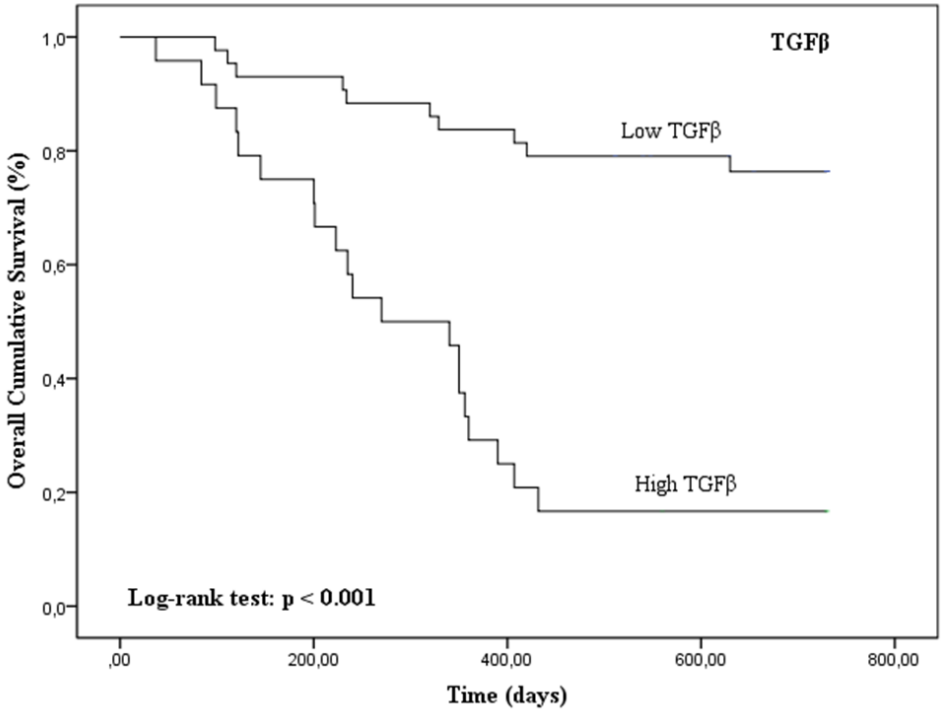

Figure 10 - Kaplan-Meier OS curves comparing TGFβ class in 67 dogs with malignant mammary tumors.

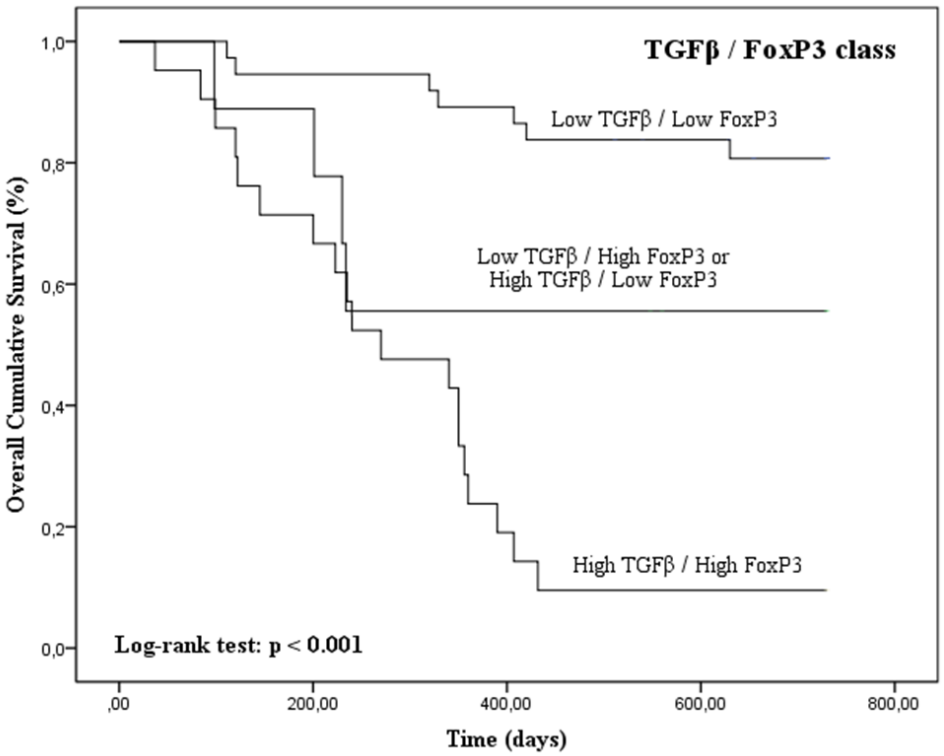

Figure 11 - Kaplan-Meier OS curves comparing TGFβ/FoxP3 class in 67 dogs with malignant mammary tumors.

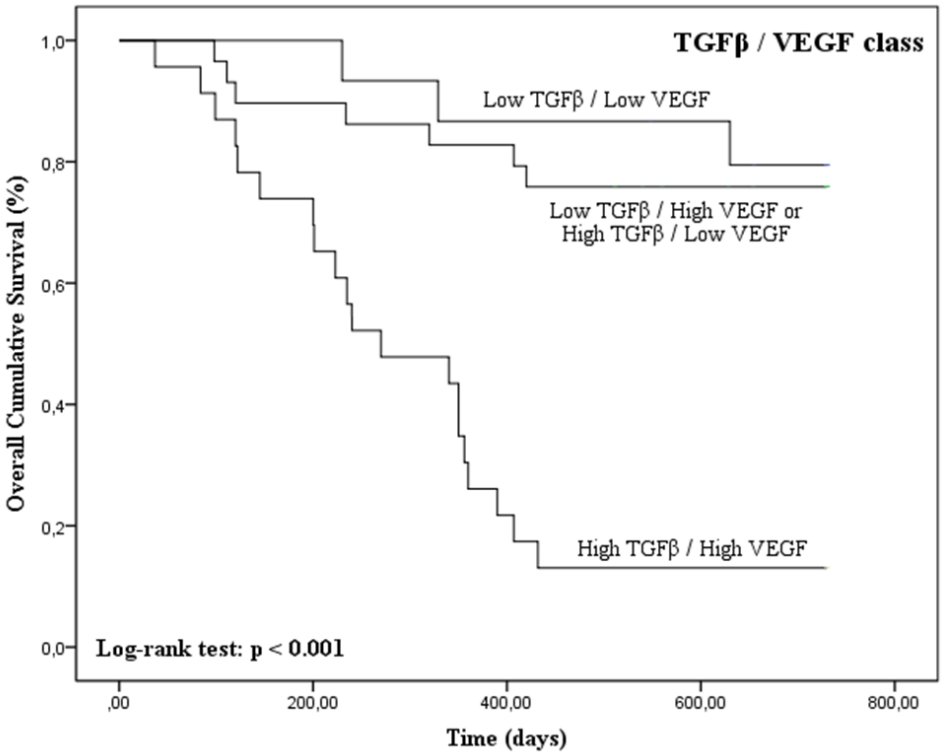

Figure 12 - Kaplan-Meier OS curves comparing TGFβ/VEGF class in 67 dogs with malignant mammary tumors.

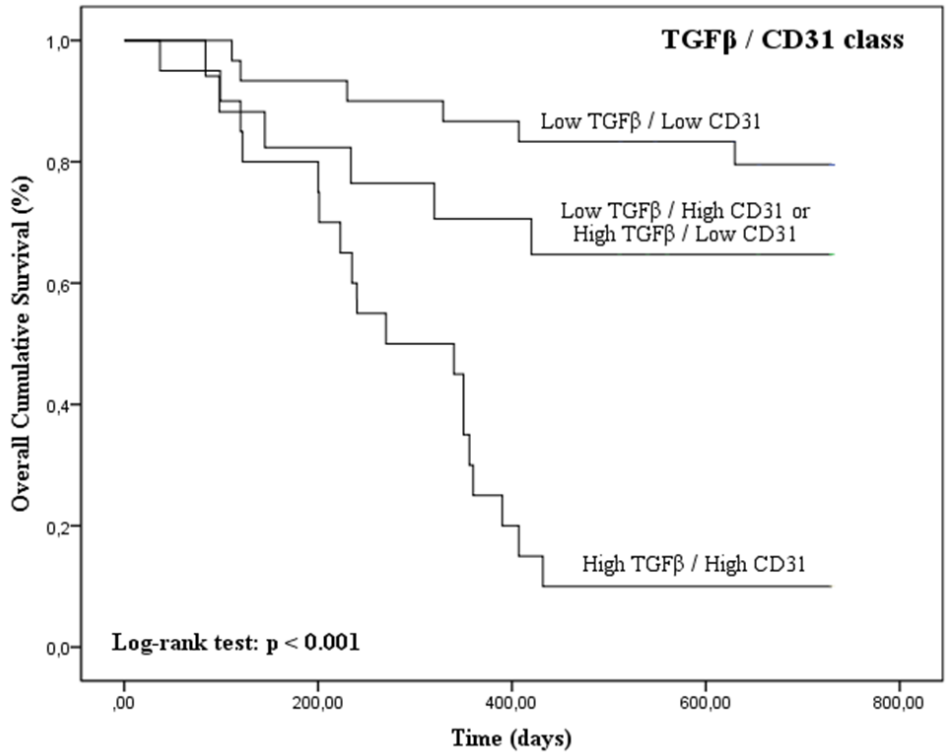

Figure 13 - Kaplan-Meier OS curves comparing TGFβ/CD31 class in 67 dogs with malignant mammary tumors.

In human breast cancer high levels of TGFβ are observed in advanced carcinomas, and have been correlated with disease progression and worse clinical outcomes [16, 18, 36].

TGFβ produced by tumor cells may act in a paracrine mode on tumor stromal cells, tumor neovessels and immune cells, contributing to tumor immunosuppression, angiogenesis and progression [14, 17].

In veterinary literature, to the best of our knowledge, the prognostic value and the role that TGFβ may have on CMT immunosuppression and angiogenesis were not investigated yet.

The present study revealed a relationship between high levels of TGFβ and presence of skin ulceration, presence of tumor necrosis, high mitotic index, marked nuclear pleomorphism, poor differentiation of tumors, high HGM, presence of neoplastic intravascular emboli and presence of lymph node metastases. The findings of our work are in accordance with recent literature in human breast cancer [10, 15-18] and suggests a link between TGFβ and more aggressive tumor phenotypes, reflecting its involvement in CMT malignant transformation. In veterinary field, one study demonstrated that, in a CMT cell line, TGFβ prompt an induction of the mesenchymal marker vimentin, increasing the invasiveness capacity of tumor cells, which is a crucial step in metastasis formation. Interestingly this induction is reversed after prolonged stimulation with TGFβ in a phenomenon similar to the mesenchymal-epithelial transition (the reverse phenomenon of epithelial-mesenchymal transition) which is useful for the formation of new tumor masses at the side of metastatic lesions [23]. These results corroborate our work and the lack of additional studies in CMT hampers more concise comparisons.

Our data demonstrated also that TGFβ levels were positively correlated with intratumoral FoxP3, VEGF and CD31. Moreover tumors with concurrent high expression of TGFβ/FoxP3, TGFβ/VEGF and TGFβ/CD31 markers were associated with parameters of tumor malignancy. Interestingly tumors with abundant TGFβ and with concurrent high expression of TGFβ/FoxP3, TGFβ/VEGF and TGFβ/CD31 were associated with shorter OS time and TGFβ/FoxP3 class retained the association with worse survival in multivariate analysis, arising as an independent predictor of poor prognosis.

Concordantly with our findings, in human breast cancer TGFβ has an important role on tumor microenvironment switch, promoting increased angiogenic activity and suppressed immune surveillance which contribute for tumor development, progression and poor clinical outcome [10, 11, 15, 22, 37]. The TGFβ in breast tumor sites acts as an important immunosuppressant repressing effector T cells anti-tumor activity [19]. Additionally, TGFβ signaling in T-cells participates in the expression and the stabilization of transcription factor FoxP3. The increasingly high concentrations of TGFβ secreted by

tumor cells induce FoxP3 expression in peripheral $CD4^+CD25^-$ T-cells and their precursors, rendering them inactive [3, 20]. This occurrence is clinically relevant since the enrichment of $CD4^+CD25^+FoxP3^+$ Treg cells in human mammary tumors is associated with poor prognosis [11]. Moreover Treg cells increase the TGFβ effects creating a positive auto-regulatory loop of TGFβ signaling in $CD4^+CD25^-$ T-cells that possibly stabilizes their regulatory phenotype [21]. FoxP3 Treg cells, in this process, needs greater attention, not only for being an important source of TGFβ but also for directly instructing cancer cells by secreting TGFβ [38]. In humans the TGFβ and FoxP3 common signaling pathways have a crucial impact in several phases of mammary carcinogenesis, including tumor angiogenic switch [11, 19, 20]. Similarly to our results, recent data in human breast cancer demonstrated that intratumoral FoxP3 was correlated with levels of TGFβ, VEGF and tumor microvessel density [11]. TGFβ and FoxP3 are reported to regulate tumor new blood vessels formation by a combination of responses that increase the production of VEGF (Donovan et al., 1997; Gupta et al., 2007) [37].
In dog mammary tumors was demonstrated that Treg cells may contribute to increased angiogenesis [24]. Another study showed that $FoxP3^+CD4^+$ T cells in dogs could be expanded *in vitro* after the addition of TGFβ and IL-2 and by TCR activation [25]. However, to the best of our knowledge, this is the first study that demonstrate the prognostic value of TGFβ. Interestingly our results suggest that in CMT may exist an autocrine/paracrine TGFβ/FoxP3 signaling loop which seems to be important as independent prognostic factor. TGFβ and Treg cells common pathways provides the tumor with a mechanism that facilitate evasion of immune surveillance and prompt the VEGF-dependent angiogenesis, contributing to CMT progression and aggression.

Conclusion

Our data suggest that the feedback-loop between TGFβ and FoxP3 regulatory T-cells may induce overexpression of VEGF and may contribute to the augmentation of certain aspects of tumor malignant phenotype and shorter OS time. Interestingly, the results of this study show that TGFβ/FoxP3 class retained their significant relationship with OS by multivariate analysis arising as an independent predictor of poor clinical outcome in malignant CMT.

Table 5 - Relationship of TGFβ/FoxP3, TGFβ/VEGF and TGFβ/CD31classes with clinicopathological variables of tumor aggressiveness.

Molecular markers		Variables of tumor aggressiveness									
		HGM				Neoplastic intravascular emboli			Lymph node metastasis		
		I	II	III	p	Absent	Present	p	Absent	Present	p
TGFβ/FoxP3	Low TGFβ / Low FoxP3	15	16	6	**<0.001**	35	2	**<0.001**	32	1	**<0.001**
	Low TGFβ / High FoxP3 or High TGFβ / Low FoxP3	2	2	5		3	6		4	5	
	High TGFβ / High FoxP3	0	0	21		8	13		3	18	
TGFβ/VEGF	Low TGFβ / Low VEGF	6	8	1	**<0.001**	14	1	**0.001**	13	2	**<0.001**
	Low TGFβ / High VEGF or High TGFβ / Low VEGF	11	9	9		23	6		22	7	
	High TGFβ / High VEGF	0	1	22		9	14		4	19	
TGFβ/CD31	Low TGFβ / Low CD31	12	15	3	**<0.001**	28	2	**<0.001**	27	3	**<0.001**
	Low TGFβ / High CD31 or High TGFβ / Low CD31	5	3	9		11	6		9	8	
	High TGFβ / High CD31	0	0	20		7	13		3	17	

n, number of samples; p, statistical significance; NS, not significant.

Table 6 - Association between considered molecular markers classes and overall survival.

Molecular markers		n	Overall Survival Univariate (Mean values)	p	Overall Survival Multivariate* (Hazard ratio)	p
TGFβ	Low TGFβ	43	627.250	**<0.001**	-	NS
	High TGFβ	24	328.375			
TGFβ/FoxP3	Low TGFβ / Low FoxP3	37	654.654	**<0.001**	**9.731** (CI: 3.982 - 23.780)	**<0.001**
	Low TGFβ / High FoxP3 or High TGFβ / Low FoxP3	9	490.333			
	High TGFβ / High FoxP3	21	296.190			
TGFβ/VEGF	Low TGFβ / Low VEGF	15	662.711	**<0.001**	-	NS
	Low TGFβ / High VEGF or High TGFβ / Low VEGF	29	612.759			
	High TGFβ / High VEGF	23	310.913			
TGFβ/CD31	Low TGFβ / Low CD31	30	644.445	**<0.001**	-	NS
	Low TGFβ / High CD31 or High TGFβ / Low CD31	17	548.882			
	High TGFβ / High CD31	20	309.600			

n, number of samples; p, statistical significance; NS, not significant; CI, confidence interval; *Multivariate Cox Proportional Hazard Analysis (Cox Regression) of overall survival (OS) in dogs with malignant mammary tumors in a prospective study with 2 years of follow-up.

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II.3 Assessing the Interleukin 35 immunoexpression in Malignant Canine Mammary Tumors: association with Clinicopathological Parameters and Prognosis

Abstract

Interleukin (IL)-35 has a prominent immunosuppressive role and its overexpression has been presented in human breast cancer. However, the impact of IL-35 in canine mammary carcinogenesis is not addressed yet. In the present study was determined the clinicopathological significance of IL-35 immunoexpression and its correlation with overall survival (OS) of 72 malignant canine mammary tumor (CMT) patients. IL-35 overexpression was associated with: skin ulceration ($p = 0.042$), tumor necrosis ($p < 0.001$), mitotic index ($p < 0.001$), nuclear pleomorphism ($p < 0.001$), tumor differentiation ($p < 0.001$), HGM ($p < 0.001$), neoplastic intravascular emboli ($p < 0.001$) and lymph node metastasis ($p < 0.001$). Additionally IL-35 was also correlated with OS ($p < 0.001$) and retained the association with worse survival in multivariate analysis, arising as an independent predictor of poor prognosis. Our results indicate for the first time that IL-35 is associated with carcinogenesis and worse prognosis of CMT.

Introduction

Interleukin (IL)-35 is a member of the IL-12 family of cytokines. This heterodimeric cytokine is composed of the IL-12p35 subunit and the Epstein- Barr virus-induced gene 3 (Ebi3) subunit and is preferentially secreted by mouse and human regulatory T-cells (Treg cells) [1-3]. IL-35 is well documented as an anti-inflammatory cytokine implicated in the regulation of autoimmune diseases [4-6]. Previous studies revealed that IL-35, produced by Treg cells, is potent to suppress the functionality of Th1, Th17 and Th2 cells [7]. More recently, reports suggest the pivotal role of IL-35 in the pathogenesis of tumor development, malignant progression and prognosis, which attracts even more the scientific community attention [1, 2, 8-10].

Recent mouse experiments have shown that IL-35 produced by Treg cells and cancer cells promotes tumor growth via enhancing myeloid cell accumulation and angiogenesis, and reducing the infiltration of activated $CD8^+$ T-cells into tumor microenvironment [11]. In general, IL-35 expression is considered to be implicated in immunosuppression along with tumor progression and poor prognosis [1, 2]. In the tumor microenvironment IL-35 induces the conversion of proliferative $FoxP3^-$ conventional T-cells into a hyporesponsive, strongly suppressive IL-35-producing $CD4^+FoxP3^-$ induced regulatory T-cell population (iTr35 cells) which has been found to potently inhibit anti-tumor T-cell responses [3, 7, 10]. This iTr35 cells population, which mediate immune suppression via IL-35 was strongly suppressive and stable *in vivo*, did not express FoxP3, did not require the other key suppressive cytokines (IL-10 or transforming growth factor-β, TGFβ) for conversion and was distinct from the known induced regulatory populations of TGF-β–iTr cells and IL-10–iTr cells which require longer conversion protocols, multiple cell types and/or additional molecules for optimal generation [3, 10]. Summarizing IL-35 is a Treg cell–specific cytokine that is required for the maximum regulatory activity of human and mouse Treg cells under both *in vitro* and *in vivo* conditions and iTr35 cells have a highly restricted genetic signature that results in a $CD4^+FoxP3^-Ebi3^+p35^+IL\text{-}10^-TGF\text{-}\beta^-$ phenotype [7, 12].

Immunohistochemical analysis revealed that IL-35 is highly expressed in several human malignancies as: pancreas cancer, nasopharyngeal carcinoma, colorectal cancer, esophageal carcinoma, hepatocellular carcinoma, cervical carcinoma [1, 2, 13, 14].

In human breast cancer patients was observed a significant increased expression of IL-35 [10]. Furthermore Ki-67, p53 and epidermal growth factor receptor (EGFR) expression

on breast cancer tissues increased when circulating IL-23:IL-35 ratio decreased. These findings suggest that IL-35 seems to be an important indicator of breast cancer progression and prognosis [9].

While these findings in human tumors are important and stimulating, the impact of IL-35 on canine mammary tumorigenesis, cancer development and clinical outcome, is not addressed yet. In the present study, in order to clarify this question, we investigated the intratumoral IL-35 expression in malignant canine mammary tumor (CMT) by immunohistochemistry, together with several clinicopathological parameters and patients overall survival.

Materials and Methods

Patient selection and tissue sample collection

A total of 72 female dogs received for diagnosis and treatment, was included in this study. The sample collection is composed of tumors excised by surgery from patients with malignant mammary cancer. None of the patients had received radiotherapy or chemotherapy.The clinical stage of animals was categorized into: local (without lymph node involvement), regional (metastasis at regional lymph nodes) and distant (presence of distant metastases) [15, 16] by the modified pathology tumor-node-metastasis TNM system [17]. On each tumor sample was also evaluated the tumor size (T1 <3 cm; T2 ≥ 3 and <5 cm; T3 ≥ 5 cm) and the skin ulceration.

Histopathological examination

Tissue sections (4-μm) were prepared from paraffin wax tissue blocks and then subjected to haematoxylin and eosin (HE) following routine methods. Each slide was assessed by two pathologists to perform the pathological classification in accordance with the criteria proposed by the World Health Organization (WHO) for CMT [18]. Tumors were also graded [mitotic index, nuclear grade, differentiation grade, histological grade of malignancy (HGM)] by the Goldschmidt and collaborators method [19]. Additional clinicopathological characteristics evaluated were presence of tumor necrosis, presence of neoplastic intravascular emboli and regional lymph node involvement.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized and rehydrated in graded concentrations of alcohol. The IL-35 immunostaining was performed with the streptavidin-biotin-peroxidase complex method using the Ultra Vision Detection System kit (Lab Vision Corporation, Fremont, CA, USA), following the manufacturer's instructions. The sections were submerged in 0.01 M, pH = 6.0, citrate antigenic retrieval buffer and subjected to pressure cooker. Then slides were incubated with anti-IL-35 antibody (EBI3, sc-32868, Santa Cruz Biotechnology, Dalla, Texas, USA; diluted to 1:200) at 4°C overnight. The antibody reaction products were observed with the chromogen 3, 3'-diaminobenzidine tetrachloride (DAB) at 0.05% with 0.01% H_2O_2 (30%). Finally, sections were counterstained with Gill's haematoxylin and viewed under a light microscope. A negative control was obtained by replacing the primary antibody with an irrelevant isotype-matched antibody. Canine lymph node and thymus sections were used as positive control.

IL-35 staining evaluation

IL-35 immunoexpression was evaluated by two observers in a blinded process to the clinical status of the patients. The staining evaluation was based on a semiquantitative method adapted from previous published studies [1, 2]. This method was scored according to the percentage of positive cancer cells (immunolabelling extension) and staining intensity. The percentage of positivity was scored as 0 (0-25% positive cells), 1 (26-50% positive cells), 2 (51-75% positive cells), 3 (> 75% positive cells). The staining intensity was scored as 0 (no staining), 1 (weakly stained), 2 (moderately stained), and 3 (strongly stained). A final score was obtained by the product of multiplication between staining intensity and the percentage of positive cells. A final immunostaining score ≤ 2 indicates a low IL-35 class, whereas a final immunohistochemical score > 2 was taken as a high IL-35 class.

Follow-up study

Patients whose cause of death remained unknown were excluded from our study. Informed consent on the collection of samples and the clinical follow-up was obtained from each patient owner. The clinical follow-up of the female dogs included in this study comprised an examination 15 days after surgery and every 90 days thereafter for a

maximum period of 730 days. The clinical follow-up examination of the patients included a physical checkup, a radiological evaluation of the thorax and an abdominal ultrasound scan. For the animals that died within the 730 days period, the overall survival (OS) time was determined from the date of surgery to the date of animal death/euthanasia in consequence to the advanced stages of the disease. For the dogs that survived more than 730 days the OS time was calculated from the date of surgery to the last clinical examination.

Statistical analysis

All statistical analysis were carried out using SPSS software (Statistical Package for the Social Sciences, Chicago, IL, USA) version 19.0. The chi-square test was performed to analyze the association between the clinicopathological characteristics and IL-35 immunoexpression. Survival curves were obtained with Kaplan-Meier method and the log-rank test was used for the survival estimates. Cox proportional hazard model for multivariate analysis was also performed. In all cases, $p < 0.05$ denotes the presence of a statistically significant difference.

Results

Clinicopathological data

According to the WHO criteria, the 72 tumors included in this work were histologically classified as: tubulopapillary carcinomas (n = 37; 51.4%), solid carcinomas (n = 5; 6.9%), complex carcinomas (n = 14; 19.4%), carcinomas in benign tumor (n = 2; 2.8%), anaplastic carcinomas (n = 2; 2.8%) and carcinosarcomas (n = 12; 16.7%). Based on the modified pathology tumor-node-metastasis (TNM) stage system, 44 patients (61.1%) were in local stage, 28 patients (38.9%) were in regional stage and there aren´t any patients in distant stage. All female dogs were free from distant metastasis at the time of diagnosis (confirmed throughout the realization of thorax X-ray and abdominal ultrasound). The HGM was classified as I (n = 18; 25%), II (n = 17; 23.6%), or III (n = 37; 51.4%) and twenty one cases (29.2%) demonstrated presence of neoplastic intravascular emboli.

Expression of IL-35 in malignant CMT

The IL-35 immunoexpression appeared as a brown color present in the cytoplasm of the neoplastic cells in a diffuse or granular pattern. The lymphocytes present in the tumor area revealed a weak staining, however only tumor cells were considered in the immunoreactivity evaluation method. The staining intensity of the adnexal normal mammary gland was always weak than the tumor. The IL-35 immunostaining was predominant in the epithelial cells in a diffuse manner (**Figure 14A**). In tumors diagnosed as carcinosarcomas, in addition to the epithelial staining, was observed intense positivity in the mesenchymal component (**Figure 14B**). In complex carcinomas the myoepithelial area was negative or revealed a weak focal positivity (**Figure 14C**). The carcinomas in

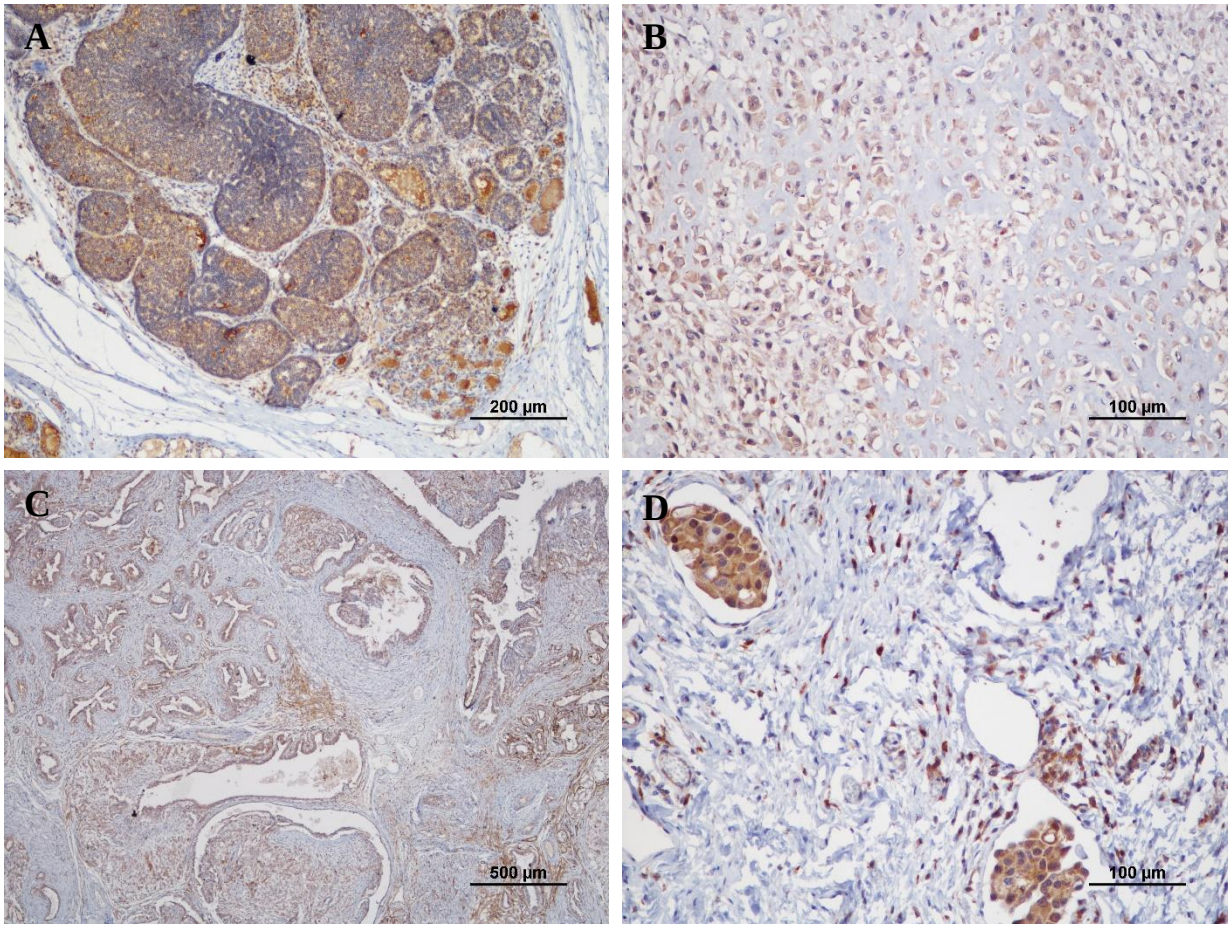

Figure 14 – Immunoreactivity for IL-35 in **(A)** solid carcinoma, note the predominant staining in the epithelial cells in a diffuse manner, bar = 200 µm; **(B)** carcinosarcoma, note the intense positivity in the mesenchymal component, bar = 100 µm; **(C)** complex carcinoma. The myoepithelial area was negative or revealed a weak focal positivity, bar = 500 µm and **(D)** neoplastic intravascular emboli. The labelling intensity of IL-35 in primary tumor and neoplastic intravascular emboli was similar, bar = 100 µm.

benign tumor were negative in the benign tumor section comprising bone or cartilage. The labelling intensity of IL-35 in primary tumor and in neoplastic intravascular emboli was similar (**Figure 14D**). Regarding IL-35 immunolabelling extension, briefly 9

(12.5%) cases showed extension 0 (0-25% positive cells), 23 (31.9%) cases showed extension 1 (26-50% positive cells) and 20 cases (27.8%) was found both in extension 2 (51–75% positive cells) and 3 (>75%). For IL-35 labelling intensity, there was a relatively homogeneous distribution between the three classes: weak (n = 19; 26.4%), moderate (n = 25; 34.7%) and strong intensity (n = 28; 38.9%).

Associations of IL-35 immunostaining with clinicopathological variables

To evaluate the clinical significance of IL-35 expression in malignant CMT, its association with several clinicopathological variables was studied. Our analysis has identified an association between the presence of aggressive disease and high IL-35 immunoexpression. As summarized in the **Table 7**, tumors with higher levels of IL-35 were associated with skin ulceration ($p = 0.042$), tumor histological type ($p < 0.001$), tumor necrosis ($p < 0.001$), high mitotic index ($p < 0.001$), marked nuclear pleomorphism ($p = 0.001$), poor tumor differentiation ($p < 0.001$), high HGM ($p < 0.001$), presence of neoplastic intravascular emboli ($p < 0.001$) and presence of lymph node metastasis ($p < 0.001$).

Follow-up study

The high intratumoral IL-35 expression was associated with poor clinical outcome ($p < 0.001$ in Kaplan-Meier curves; **Figure 15**). Animals with high intratumoral IL-35 immunostaining demonstrated a lower OS time (n = 42; 390,65 ± 36,27 days) than animals who presented low intratumoral IL-35 expression (n = 30; 686,02 ± 27,57 days). The clinicopathological parameters related to shorter OS, included: tumor size ($p = 0.034$); skin ulceration ($p = 0.033$); nuclear grade ($p < 0.001$); differentiation grade ($p < 0.001$); mitotic index ($p < 0.001$); HGM ($p < 0.001$); tumor necrosis ($p = 0.001$); lymph node metastasis ($p < 0.001$) and neoplastic intravascular emboli ($p < 0.001$). The multivariate analysis performed with Cox proportional hazard model demonstrated that high mitotic index and high intratumoral IL-35 expression were independent predictors of OS ($p < 0.001$ and $p = 0.029$, respectively). More information is provided in **Table 8**.

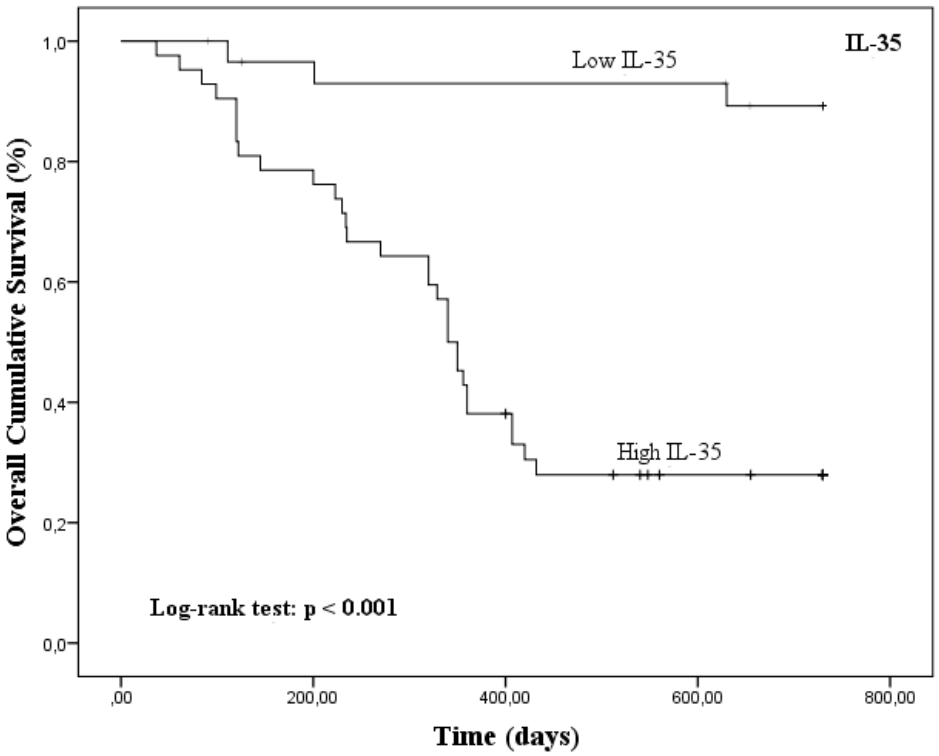

Figure 15 - Kaplan-Meier OS curves comparing IL-35 class in 72 dogs with malignant mammary tumors.

Discussion

IL-35 is well documented as an anti-inflammatory cytokine implicated in the regulation of autoimmune diseases [4-6]. However the IL-35 production in the tumor microenvironment may contribute to tumor progression and tumor immune surveillance. Recently, IL-35 overexpression has been described to be involved in the development, progression and poor prognosis of several human tumors, including breast cancer [1, 2, 9, 10, 13].

In human breast cancer patients was observed a significant increased expression of IL-35 [10] and findings suggest that IL-35 seems to be an important indicator of breast cancer progression [9]. In CMT, to the best of our knowledge, this is the first study to investigate the role of IL-35 in canine mammary tumorigenesis, cancer development and clinical outcome.

In the present study, we have observed that IL-35 staining was localized in the cytoplasm of cancer cells. Moreover, IL-35 was highly expressed in cancer cells compared with adnexal non-tumoral mammary gland, indicating that this interleukin might be involved in the pathogenesis of mammary cancer. Immunohistochemistry assay demonstrated that

IL-35 higher expression was significantly associated with more aggressive tumor phenotypes: presence of skin ulceration, presence of tumor necrosis, high mitotic index, marked nuclear pleomorphism, poor tumor differentiation, high HGM, presence of neoplastic intravascular emboli and presence of lymph node metastasis.

Our results showed that, in CMT, IL-35 overexpression was significantly associated with advancement of tumor stage and these findings were in agreement with several reports in human malignancies. In human lung cancer cells, siRNA silencing of Ebi3 inhibits cancer cell proliferation, whereas stable expression of Ebi3 in lung cancer cells confers growth promoting activity *in vitro* [20]. In human colorectal cancer and in nasopharyngeal carcinoma IL-35 levels were highly correlated to the severity of malignancy and the clinical stage of tumors [1, 2].

In human breast cancer was also described an increased expression of IL-35. Interestingly Ki-67, p53 and EGFR expression increased when circulating IL-23:IL-35 ratio decreased, suggesting that IL-35 seems to be an important indicator of cancer progression [9, 10].

To better elucidate the clinical significance of IL-35 in CMT, we further studied the impact of IL-35 on the overall survival of mammary cancer patients. Interestingly, as described in studies in human cancers [1, 2, 11], IL-35 overexpression was correlated with lower OS of animals. Moreover, in our study, multivariate analysis demonstrated that high mitotic index and high intratumoral IL-35 expression were independent predictors of prognosis.

IL-35 is a Treg cell-secreted cytokine that inhibits T cell proliferation and function [7]. Treg cells suppress other immune effector cells by numerous mechanisms, one of which is the secretion of inhibitory cytokines [3, 21, 22]. In tumor microenvironment IL-35 induces the conversion of conventional T-cells into a suppressive IL-35-producing $CD4^{+}Foxp3^{-}$ induced regulatory T-cell population (iTr35 cells) which can contribute to the enhancement of tumor growth [3, 6, 7]. Additionally Treg cell-derived IL-35 promoted the expression of multiple inhibitory receptors, thereby facilitating intratumoral T-cell exhaustion, limiting anti-tumor immunity and contributing to T-cell dysfunction in the tumor microenvironment [12]. The intratumoral expression of IL-35 leads to reduced T-cells cytotoxic activities and decreased survival of the immunocompetent cells with anti-tumor properties [11, 12].

Although Treg cells were described as the primary source of IL-35, recent studies in human prostate cancer have also suggested that $CD8^{+}$ Tregs may exploit IL-35 as a dominant regulatory mechanism [23]. Furthermore, regulatory B cells have recently been

shown to produce IL-35 [24, 25]. Hence, the inflammatory cells in tumor microenvironment facilitate the enrichment of an IL-35$^+$ Treg population which seems to be one of the mechanisms for tumor immune evasion [12].

Our data suggest that, in CMT, similar mechanism could be present and demonstrated for the first time that IL-35 is involved in CMT carcinogenesis and worse clinical outcome, arising as an independent predictor of poor prognosis in these tumors.

Conclusion

The present study evaluated the immunohistochemical pattern of IL-35 in malignant CMT and its association with clinicopathological parameters and OS. Our data suggests that IL-35 is involved in malignant progression of mammary cancer in dogs. Moreover, high IL-35 expression was correlated with unfavorable prognosis by univariate and multivariate analysis. Therefore, IL-35 may be a new biomarker for the prognosis of CMT patients.

Table 7 - Relationship between IL-35 expression and clinicopathological parameters in malignant CMT.

Clinicopathological parameters	Low IL-35 n	High IL-35 n	p
Tumor size			
T1 <3cm	11	13	
T2 ≥3cm and <5cm	9	16	NS
T3 ≥5 cm	10	13	
Skin ulceration			
Absent	24	26	**0.042**
Present	6	16	
Histological type			
Tubulopapillary C.	17	20	
Solid C.	0	5	
Complex C.	11	3	**<0.001**
Carcinoma in benign tumor	2	0	
Anaplastic C.	0	2	
Carcinosarcoma	0	12	
Tumor necrosis			
Absent	21	10	**<0.001**
Present	9	32	
Mitotic index			
I	15	4	**<0.001**
II	13	14	
III	2	27	
Nuclear grade			
I	1	0	**<0.001**
II	21	7	
III	8	35	
Differentiation grade			
I	14	4	**<0.001**
II	13	4	
III	3	34	
HGM			
I	16	1	
II	17	1	**<0.001**
III	10	22	
Neoplastic intravascular emboli			
Absent	29	22	**<0.001**
Present	1	20	
Lymph node metastasis			
Absent	27	17	**<0.001**
Present	3	25	

n, number of samples; p, statistical significance; C, carcinoma; NS, not significant.

Table 8 - Relationship of clinicopathological variables and IL-35 expression with overall survival.

Clinicopathological parameters	n	Overall Survival Univariate (Mean values)	p	Overall Survival Multivariate* (Hazard ratio)	p
Tumor size					
T1 <3cm	24	612.292			
T2 ≥3cm and <5cm	25	441.533	**0.034**	—	NS
T3 ≥5 cm	23	471.411			
Skin ulceration					
Absent	50	550.068	**0.033**	—	NS
Present	22	419.520			
Histological type					
Tubulopapillary C.	37	564.138			
Solid C.	5	276.000			
Complex C.	14	674.071			
Carcinoma in benign tumor	2	440.000	**<0.001**	—	NS
Anaplastic C.	2	118.500			
Carcinosarcoma	12	266.667			
Tumor necrosis					
Absent	31	629.824	**0.001**	—	NS
Present	41	423.797			
Mitotic index					
I	19	623.667		**4.080**	
II	27	563.926	**<0.001**	(CI: 1.998 – 8.331)	**<0.001**
III	26	283.692			
Nuclear grade					
I	1	730.000			
II	28	621.286	**<0.001**	—	NS
III	43	383.907			
Differentiation grade					
I	8	673.571			
II	22	602.773	**<0.001**	—	NS
III	42	377.429			
HGM					
I	18	653.882			
II	17	612.941	**<0.001**	—	NS
III	37	332.216			
Neoplastic Intravascular emboli					
Absent	51	593.531	**<0.001**	—	NS
Present	21	309.702			
Lymph node metastasis					
Absent	44	637.545	**<0.001**	—	NS
Present	28	319.036			
Intratumoral IL-35					
Low	30	686.019	**<0.001**	**4.244**	
High	42	390.652		(CI: 1.160 – 15.525)	**0.029**

n, number of samples; p, statistical significance; NS, not significant; CI, confidence interval; *Multivariate Cox Proportional Hazard Analysis (Cox Regression) of overall survival (OS) in dogs with malignant mammary tumors in a prospective study with 2 years of follow-up.

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Chapter III – Relationship of T-Lymphocytes and Macrophages with Emergent Molecular Targets

Original Research Articles:

High COX-2 expression is associated with increased Angiogenesis, Proliferation and Tumoral Inflammatory Infiltrate in Canine Malignant Mammary Tumors: a Multivariate Survival Study.

Maria Isabel Carvalho, Isabel Pires, Justina Prada, Teresa Raposo, Hugo Gregório, Luís Lobo, Felisbina Luísa Queiroga.

Veterinary and Comparative Oncology, 2016

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Positive Interplay between $CD3^{+}$ T-lymphocytes and Concurrent COX-2/EGFR Expression in Canine Malignant Mammary Tumors.

Maria Isabel Carvalho, Isabel Pires, Justina Prada, Adriano Fernandes Ferreira, Felisbina Luísa Queiroga.

Anticancer Research, 2015

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Intratumoral $CD3^{+}$ T-lymphocytes immunoexpression and its association with c-kit, Angiogenesis and Overall Survival in Malignant Canine Mammary Tumors.

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Analytical Cellular Pathology, 2015

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III.1 High COX-2 expression is associated with increased Angiogenesis, Proliferation and Tumoral Inflammatory Infiltrate in Canine Malignant Mammary Tumors: a Multivariate Survival Study

Abstract

COX-2 expression affects mammary tumorigenesis by promoting angiogenesis and cell proliferation, encouraging metastatic spread and tumor associated inflammation. Samples of canine mammary tumors (n = 109) were submitted to immunohistochemistry to detect COX-2, CD31, VEGF, Ki-67, CD3 and MAC387 expression. Concurrent high expression of COX-2/CD31, COX-2/VEGF, COX-2/Ki-67, COX-2/CD3 and COX-2/MAC was associated with elevated grade of malignancy, presence of intravascular emboli and presence of lymph node metastasis. Tumors with high COX-2 ($p < 0.001$) and tumors with concurrent expression of high COX-2 and high CD31 ($p = 0.008$); high VEGF ($p < 0.001$); high Ki-67 ($p < 0.001$); high $CD3^+$ T-lymphocytes ($p = 0.002$) and elevated MAC387 macrophages ($p = 0.024$) were associated with shorter overall survival (OS) time. Interestingly the groups with high COX-2/CD31 and high COX-2/VEGF retained their significance after multivariate analysis arising as independent predictors of OS. Present data highlight the importance of COX-2 in canine mammary tumorigenesis.

Introduction

COX-2 is the inducible form of the enzyme catalyzing the rate-limiting step in prostanoid biosynthesis, which is renowned for its participation in tumor progression and spread [1]. COX-2/prostaglandin E2 (PGE2) pathway plays an important role in carcinogenesis, due to the participation in several cancer hallmarks [2]. Changes in COX-2 expression have been observed in mammary tumors and related to mammary tumorigenesis, both in humans [3-5] and dogs [6, 7]. In both species, COX-2/PGE2 overexpression appears to be related with angiogenesis, inducing pro-angiogenic factors and enhancing endothelial cells motility and vascular hyperpermeability [6, 8]. Moreover, the tumor cell proliferation and differentiation can be modulated through multiple signaling pathways headed by PGE2 in autocrine and paracrine mode [2, 9, 10]. Despite the large knowledge on COX-2 carcinogenic pathways in human breast cancer, its molecular contribution in canine mammary tumors (CMT) pathogenesis is still poorly understood. Furthermore, the prognostic impact of combined association between COX-2 expression and molecular markers of angiogenesis and proliferation was not studied yet nor in humans, neither in dogs.

Another hallmark of cancer which COX-2 showed share common signaling pathways is the inflammation. Although epidemiological and experimental evidence strongly implicates chronic inflammation as a risk factor for cancer, the mechanisms by which inflammation and inflammatory mediators result in neoplastic transformation and progression have not been completely determined [11-13]. Chronic inflammation induces the production of pro-inflammatory mediators, including prostaglandins, cytokines and chemokines by stromal cells, contributing to tissue microenvironment shift from normal to aberrant [14-16]. This inflammatory microenvironment, which is associated with changes in leukocyte profiles and in their functionality, can initiate cell transformation and promote tumor growth, angiogenesis and metastasis [17].

In human breast cancer PGE2 has the ability to modulate functions of different immune cell populations including T-lymphocytes and macrophages [18-20]. PGE2 induce suppression of antigen-presenting dendritic cells, leading to a reduced activation of anti-tumor cytotoxic $CD8^+$ T-cells [21] and decreases the production of interferon gamma (IFNγ) and interleukin-2 (IL-2) [22]. PGE2, and possibly other COX-2-derived prostanoids induce the restraint of M1, and/or promotion of M2 macrophage function

related to carcinogenesis phenomenon [23]. Collectively, these roles of COX-2/PGE2 may allow neoplastic cells to evade attack by the immune system [2].

In the dog, to the best of our knowledge, the present study is the first that focuses on researching the prognostic impact of the combined association between COX-2 expression, angiogenesis, proliferation and inflammatory infiltrate in a large series of malignant mammary tumors. Understanding the contribution of COX-2 in the pathogenesis of CMT, as well as the underlying molecular pathways, might enlighten new therapeutic targets and inspire the creation of new safer therapeutic agents with reasonable benefit and fewer side effects.

Materials and Methods

Tissue samples

In this study were included 109 CMT. The samples for histopathology were fixed in 10% neutral buffered formalin, before being embedded in paraffin and cut in 4 µm sections, following routine methods. The histopathological diagnosis was made on haematoxylin and eosin stained sections, by the WHO classification for CMT [24] and tumors were graded in accordance with the method proposed by Goldschmidt and colleagues [25]. The clinicopathological characteristics evaluated in each sample were tumor size (T1 <3 cm; T2 ≥3 and <5 cm; T3 ≥5 cm), skin ulceration, tumor necrosis, mitotic index, nuclear grade, differentiation grade, histological grade of malignancy (HGM), neoplastic intravascular emboli and regional lymph node involvement. For this study, one tumor was selected per animal. Therefore, in case of more than one malignant tumor, the neoplasia with the most aggressive clinical (larger size, skin ulceration) and histological features (high histological grade of malignancy) was chosen as described previously [26].

Immunohistochemical analysis

For immunohistochemistry, 4 µm sections were prepared and the detection of COX-2, CD31; VEGF; Ki-67; CD3 and MAC387 was carried out by the streptavidin-biotin-peroxidase complex method with a commercial detection anti-mouse/anti-rabbit polyvalent system (Ultra Vision Detection System; Lab Vision Corporation, Fremont, USA) according the manufacturer's instructions. Antigen retrieval was performed by microwave treatment for 3x 5 min at 750W in 0.01 M citrate buffer, pH = 6.0, followed by cooling for 20 min at room temperature. All sections were incubated with the primary

specific antibody: COX-2 (Clone SP21, Transduction Laboratories, Lexington, Kentucky, USA; 1:40 dilution), CD31 (Clone JC70A, Dako, Glostrup, Denmark; 1:20 dilution), VEGF (Clone JH121, Thermo Scientific, Waltham, MA USA; 1:100 dilution), Ki-67 (Clone MIB-1, Dako, Glostrup, Denmark; 1:50 dilution) and MAC387 (Clone MCA874G, AbDSerotec, Kidlington, UK; 1:100 dilution) for 24 hours at 4ºC; CD3 (polyclonal antibody, Dako, Glostrup, Denmark, 1:50 dilution), for 2 hours at room temperature. The antibody reaction products were observed with the cromagen 3,3'-diaminobenzidine tetrachloride (DAB) at 0.05% with 0.01% H_2O_2 (30%). After a final washing in distilled water, the sections were counterstained with haematoxylin, dehydrated, cleared and mounted. The primary antibody was replaced with phosphate-buffered saline (PBS) for negative controls and this study also included adequate positive controls. The positive control used was macula densa of young dog kidney for COX-2; dog angiosarcoma for CD31; liver section for VEGF; epidermis as internal positive control for Ki-67 and sections of canine lymph nodes for CD3 and MAC387.

Quantification of immunoreactivity

For COX-2 evaluation, was used a semiquantitative method previous described [27, 28]. The percentage of COX-2–positive tumor cells was graded as 0 (0% positive cells), 1 (<10% positive cells), 2 (10–50% positive cells), 3 (51–80% positive cells), and 4 (>80% positive cells), and the intensity of COX-2 immunoreactivity was graded as 0=negative, 1=weak, 2=moderate, and 3=strong staining. Each COX-2 score represented the product of the percentage of positive tumor cells and staining intensity and ranges between 0 and 12. The final scores defined low ($\leqslant$6) or high (>6) immunoreactivity.

The assessment of microvessel density (MVD) by CD31 immunolabelling was based on a method applied to studies of angiogenesis in CMT [6, 29]. All positive cells or cell clusters were considered as a single countable microvessel. Under low-power magnification (40x), two hotspots were identified. Subsequently, under a 200x magnification the stained microvessels were counted. Three different fields were considered in each of these areas [30]. The total number of microvessels was obtained by adding the number of stained microvessels of the three different fields considered in each of the vascular hotspot areas.

The VEGF staining was scored according to a method previously used in CMT [6, 31]. Briefly, VEGF staining was classified into five different categories: '0' (no staining

detected in tumor cells); '+/-' (<10% of tumor area showed positive staining); '+' (10–25% stained), '++' (>25–50% stained) or '+++' (>51% stained). When more than 10% of the tumor cells showed positive VEGF staining, the tumor section was considered to have positive VEGF expression. To determine the Ki-67 index the area of highest labelling was searched. Ki-67 expression was evaluated by counting 1000 tumor cells with the help of a microscopic grid, at high magnification (400x) and the index was expressed as percentage, following recent recommendations [32].
For CD3 and MAC387 evaluation, cells staining positively were counted in three hotspot regions of the tumor firstly defined at low magnification, in 10 high-power fields, with 400x magnification. The number expressed for $CD3^+$ T-lymphocytes and for tumor-associated macrophages (TAMs) represents the mean value ± standard error of mean following a methodology previously used [31, 33].

Follow-up data

After surgical excision of tumors, follow-up was carried out for a mean period of 479 days (minimum 37, maximum 730 days). Overall survival (OS) was defined as the period between surgery and natural death due to the tumor or euthanasia in advanced stages of the disease (confirmed at necropsy).

Statistical analysis

The statistical software SPSS (Statistical Package for the Social Sciences) version 19.0 was used for statistical analysis. The Chi-square test was used to study the categorical variables. Analysis of variance (ANOVA) was used for analyzing continuous variables. When the variables were categorized, the mean value of each variable was used as cut-off point. Survival curves were generated by the Kaplan–Meier method and survival rates were compared using the Log rank test. Cox proportional hazard model for multivariate analysis was also performed. In all statistical comparisons, $p<0.05$ was accepted as denoting significant differences.

Results

Tumors

The samples included in this study were classified in: tubulopapillary carcinoma n = 48, complex carcinoma n = 27, solid carcinoma n = 20, anaplastic carcinoma n = 3 and carcinosarcoma n = 11. Thirty seven tumors had lymph node metastases. The infiltration of tumor cells into the vessels was observed in 35 of 109 cases. The HGM was classified as I (n = 37), II (n = 31), or III (n = 41).

COX-2; CD31; VEGF; Ki-67; $CD3^+$ T-lymphocytes and MAC387 macrophages immunostaining

The immunoreactivity for COX-2 was observed as brown color present in the cytoplasm, nuclear membrane and cytoplasmic membrane, in a diffuse and homogeneous pattern. The COX-2 staining was predominant in the epithelial cells with exception of carcinosarcomas where positivity was observed in the mesenchymal component. In tumors where positivity of COX-2 was not diffuse, the positive areas had also higher T-lymphocyte and macrophage inflammatory infiltrate. The labelling intensity of COX-2 in primary tumor and in neoplastic intravascular emboli was similar, however the extension of COX-2 staining in neoplastic intravascular emboli was always diffuse. The CD31 immunolabelling was observed in endothelial cells as a subtle outline of microvessels and occasionally in macrophages. The VEGF immunostaining was frequently observed in the cytoplasm of tumor cells and was more intense in epithelial neoplastic cells coating ducts or tubulopapillary formations with a granular pattern. Solid carcinoma areas presented predominantly strong immunoexpression of VEGF. For Ki-67 the immunoreactivity occurred always in the nucleus, appearing in a granular labeling pattern. The CD3 immunostaining was observed in the cytoplasm or/and in the cytoplasmic membrane of T-lymphocytes and the diffuse inflammation emerged as the predominant pattern of infiltration. T-lymphocytes contact closely with neoplastic cells and sometimes were accumulated in perilobular and perivascular clusters. The macrophages' staining was detected by the observation of an intense cytoplasmic and nuclear labelling. Tumor associated macrophages were distributed in clusters, surrounding ducts and in their lumen and also at the tumor periphery, sometimes infiltrating the capsule. T-lymphocytes were

located predominately in the intratumoral area, whereas macrophages were also observed frequently within the periphery of the tumor

Associations between COX-2; CD31; VEGF; Ki-67; $CD3^+$ T-lymphocytes and MAC387 macrophages

High COX-2 immunoexpression revealed a statistically significant association with CD31 ($p < 0.001$), VEGF ($p < 0.001$), Ki-67 ($p = 0.017$), $CD3^+$ T-lymphocytes ($p < 0.001$) and MAC387 macrophages ($p = 0.007$).

Associations of clinicopathological features with COX-2, CD31, VEGF, Ki-67, $CD3^+$ T-lymphocytes and MAC387 macrophages immunostaining

Tumors with high COX-2 showed significant associations with larger tumor size ($p = 0.001$), presence of skin ulceration ($p = 0.001$), tumor histological type ($p = 0.006$), higher mitotic index ($p = 0.001$), more marked nuclear pleomorphism ($p < 0.001$), less tubular formation ($p < 0.001$), elevated HGM ($p < 0.001$), presence of neoplastic intravascular emboli ($p = 0.009$) and presence of lymph node metastasis ($p = 0.002$). The CD31 immunostaining was statistically associated with skin ulceration ($p = 0.004$), tumor histological type ($p = 0.045$), tumor necrosis ($p = 0.018$), higher mitotic index ($p = 0.004$), nuclear grade ($p = 0.012$), differentiation grade ($p = 0.004$), elevated HGM ($p < 0.001$), presence of neoplastic intravascular emboli ($p = 0.01$) and presence of lymph node metastasis ($p < 0.001$). VEGF positive tumors showed significant associations with tumor size ($p = 0.002$), skin ulceration ($p = 0.014$), tumor histological type ($p = 0.035$), mitotic index ($p < 0.001$), nuclear grade ($p = 0.001$), differentiation grade ($p = 0.002$), HGM ($p < 0.001$) and presence of lymph node metastasis ($p = 0.001$). Tumors with higher proliferation index demonstrated associations with mitotic index ($p = 0.002$), nuclear grade ($p < 0.001$), differentiation grade ($p = 0.005$), HGM ($p < 0.001$), presence of neoplastic intravascular emboli ($p = 0.014$) and presence of lymph node metastasis ($p = 0.004$). Tumors with higher infiltration of T-lymphocytes showed associations with skin ulceration ($p = 0.014$), tumor necrosis ($p = 0.001$), nuclear grade ($p = 0.048$), differentiation grade ($p = 0.001$), HGM ($p = 0.002$), presence of neoplastic intravascular emboli ($p < 0.001$) and presence of lymph node metastasis ($p < 0.001$). Tumors with higher infiltration of macrophages were associated with differentiation grade ($p = 0.026$),

HGM (p = 0.004) and presence of lymph node metastasis (p = 0.011). The summary **Table 1** and **Table 2** highlight all the statistically significant associations.

Relationship of COX-2/CD31; COX-2/VEGF; COX-2/Ki-67; COX-2/CD3 and COX-2/MAC groups with clinicopathological variables of tumor aggressiveness

This study considered COX-2/CD31, COX-2/VEGF, COX-2/Ki-67, COX-2/CD3 and COX-2/MAC groups. Each group was divided in three categories: low immunoreactivity for both molecular markers; low immunoreactivity for one marker and high for other and high immunoreactivity for both molecular markers. In **Figure 1** two cases of simultaneous high or low COX-2, VEGF or CD31 immunoexpression are illustrated.
The considered groups were statistically associated with HGM (p < 0.001 for all groups), neoplastic intravascular emboli (p < 0.001 for COX-2/CD31, COX-2/VEGF and COX-2/CD3; p = 0.001 for COX-2/Ki-67. COX-2/MAC showed no association with neoplastic intravascular emboli) and lymph node metastasis (p < 0.001 for all groups). More information is provided in **Table 3.**

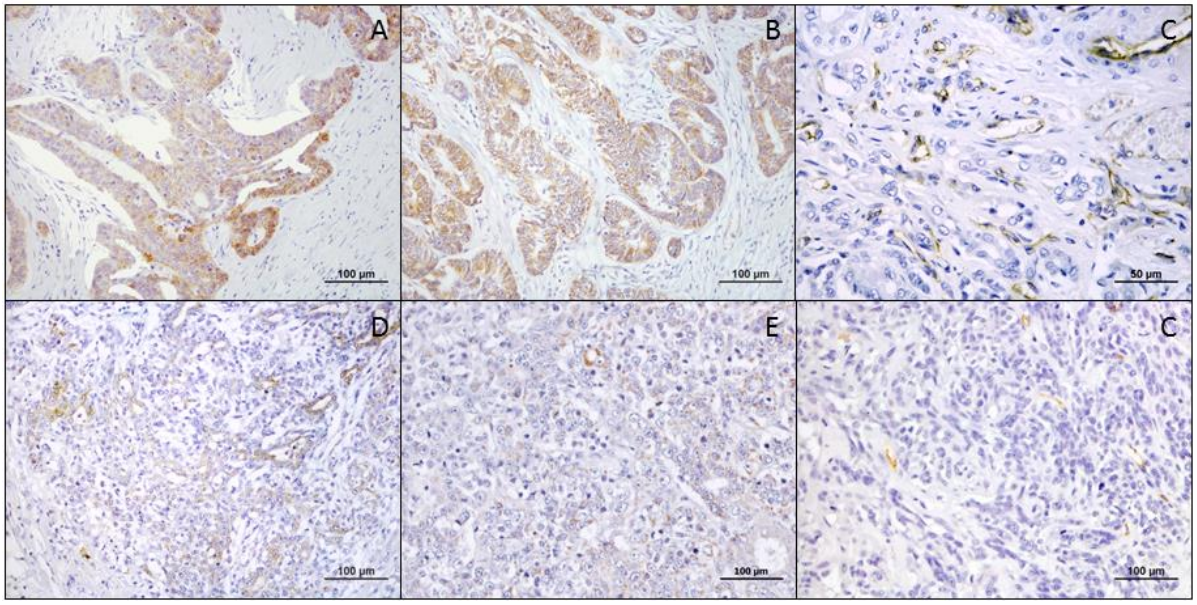

Figure 1 - Tubulopapillary carcinoma with high expression of COX-2 **(A)**; VEGF **(B)** and CD31 **(C)**. Solid carcinoma with low expression of COX-2 **(D)**; VEGF **(E)** and CD31 **(F)**.

Follow-up study

Present data (**Table 4**) showed that tumors with high COX-2 (p < 0.001) and tumors with concurrent high COX-2 and high CD31 (p = 0.008); high COX-2 and high VEGF (p < 0.001); high COX-2 and high Ki-67 (p < 0.001); high COX-2 and high $CD3^+$ T-

lymphocytes (p = 0.002); high COX-2 and high MAC387 macrophages (p = 0.024) were associated with shorter OS of animals (**Figure 2-7**).

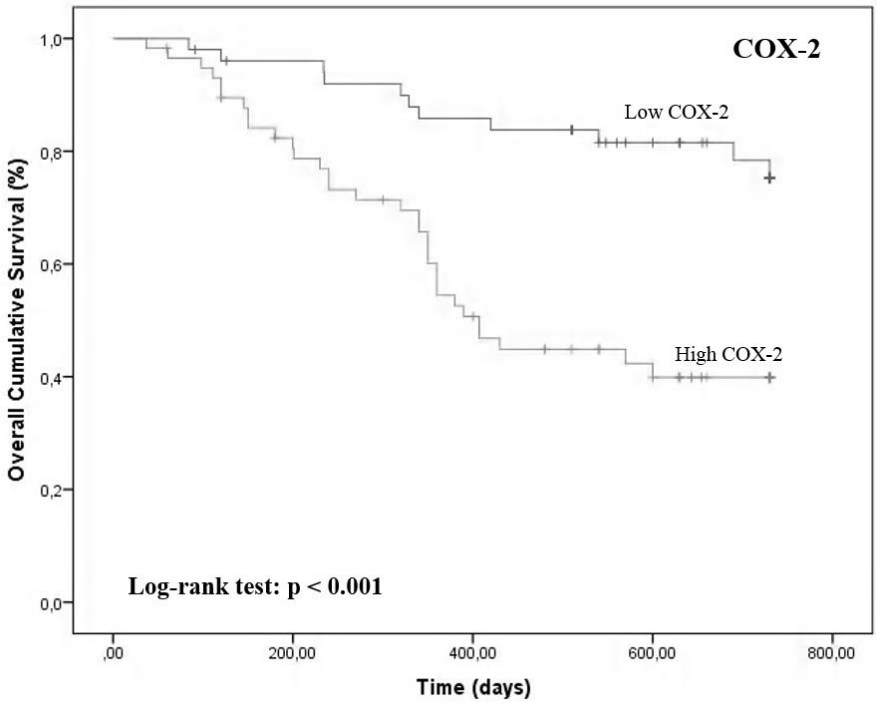

Figure 2 - Kaplan-Meier overall survival curves comparing COX-2 categories in 109 dogs with malignant mammary tumors. Female dogs with highCox-2 had significantly shorter overall survival intervals.

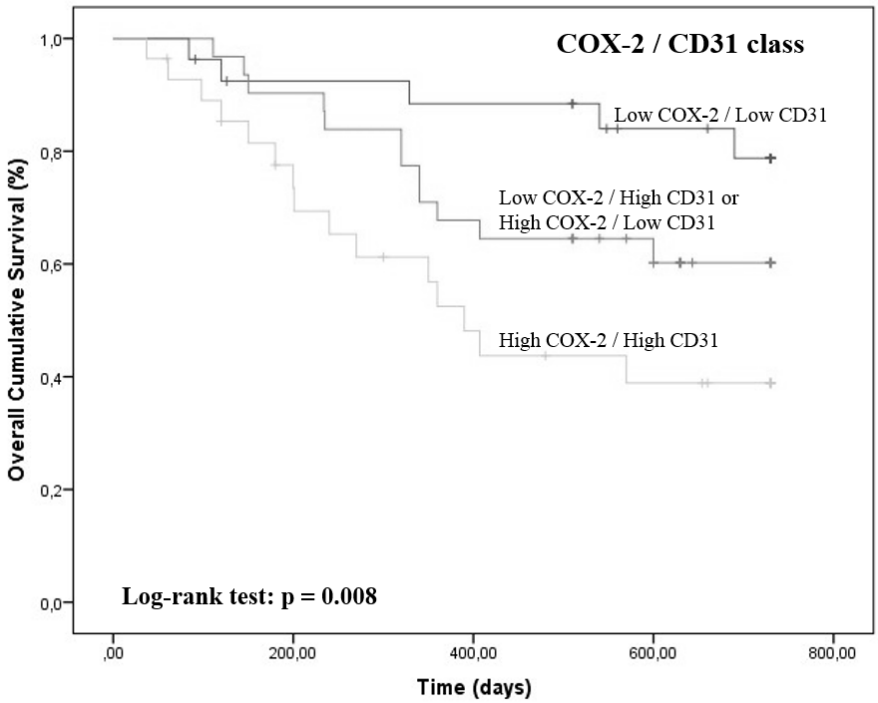

Figure 3 - Kaplan-Meier overall survival curves comparing COX-2/CD31 group categories in 109 dogs with malignant mammary tumors. Female dogs with highCox-2/highCD31 tumors had significantly shorter overall survival intervals.

The multivariate analysis performed with Cox proportional hazard model demonstrated that COX-2/CD31 [Hazard ratio (95% CI): 0.275 (0.051-1.481); p = 0.023] and COX-2/VEGF [Hazard ratio (95% CI): 11.397 (1.488-87.311); p = 0.019] groups were independent predictors of OS **(Table 4)**.

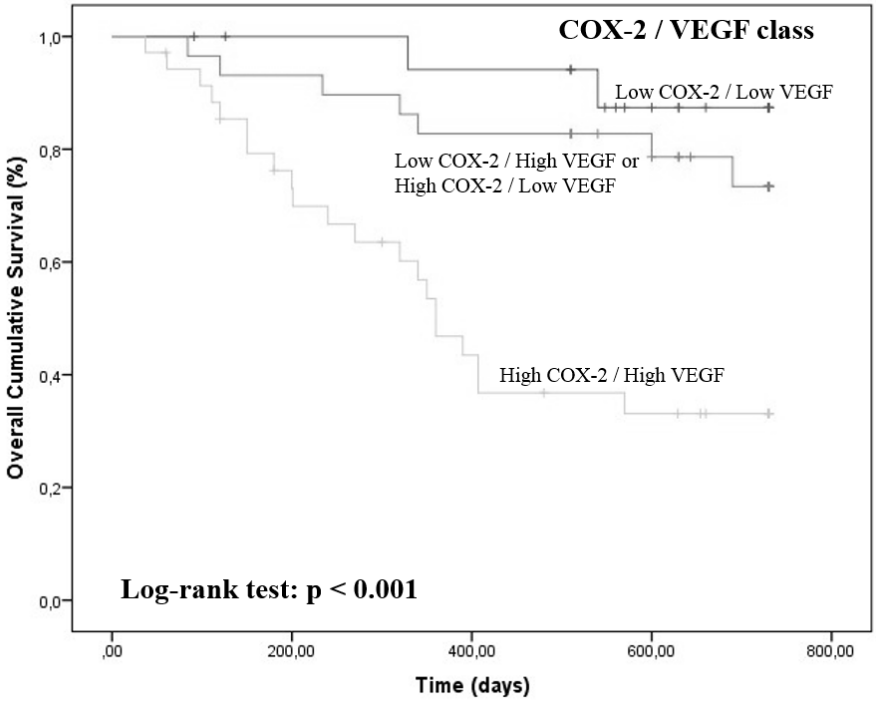

Figure 4 - Kaplan-Meier overall survival curves comparing COX-2/VEGF group categories in 109 dogs with malignant mammary tumors. Female dogs with highCox-2/highVEGF tumors had significantly shorter overall survival intervals.

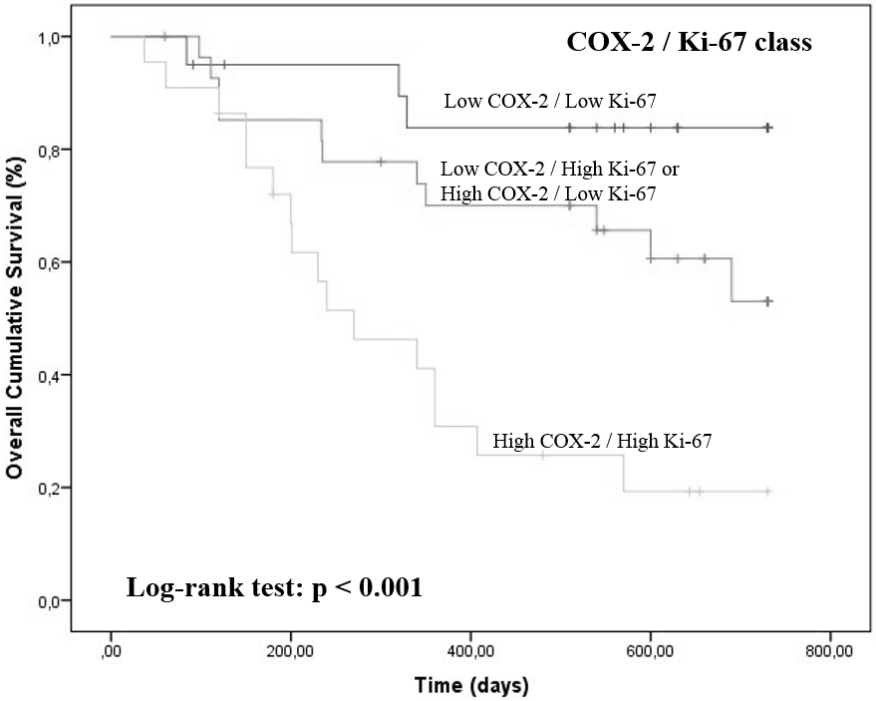

Figure 5 - Kaplan-Meier overall survival curves comparing COX-2/Ki-67 group categories in 109 dogs with malignant mammary tumors. Female dogs with highCox-2/highKi-67 tumors had significantly shorter overall survival intervals.

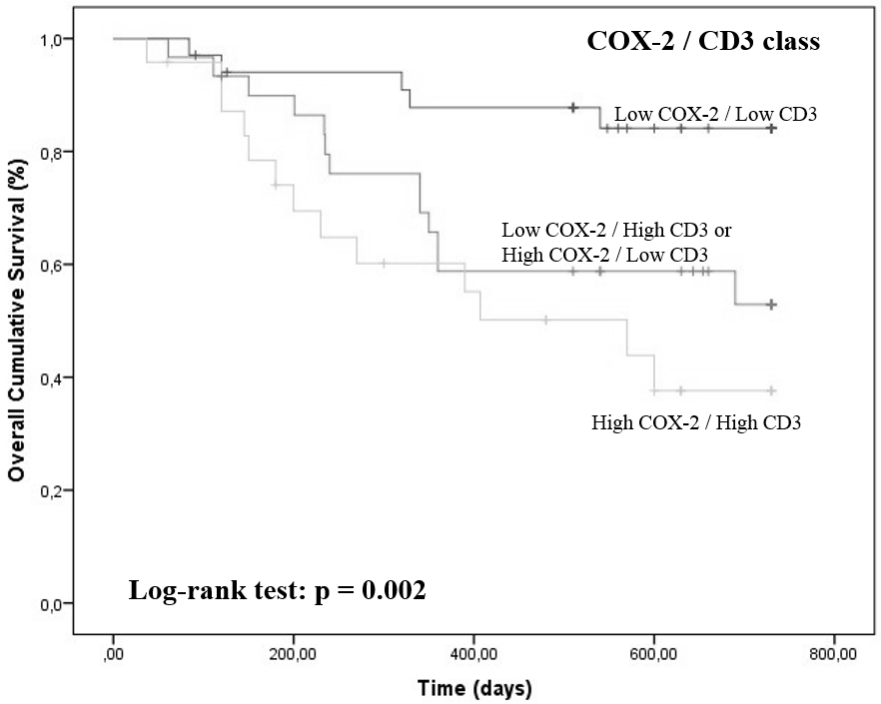

Figure 6 - Kaplan-Meier overall survival curves comparing COX-2/CD3 group categories in 109 dogs with malignant mammary tumors. Female dogs with highCox-2/highCD3 tumors had significantly shorter overall survival intervals.

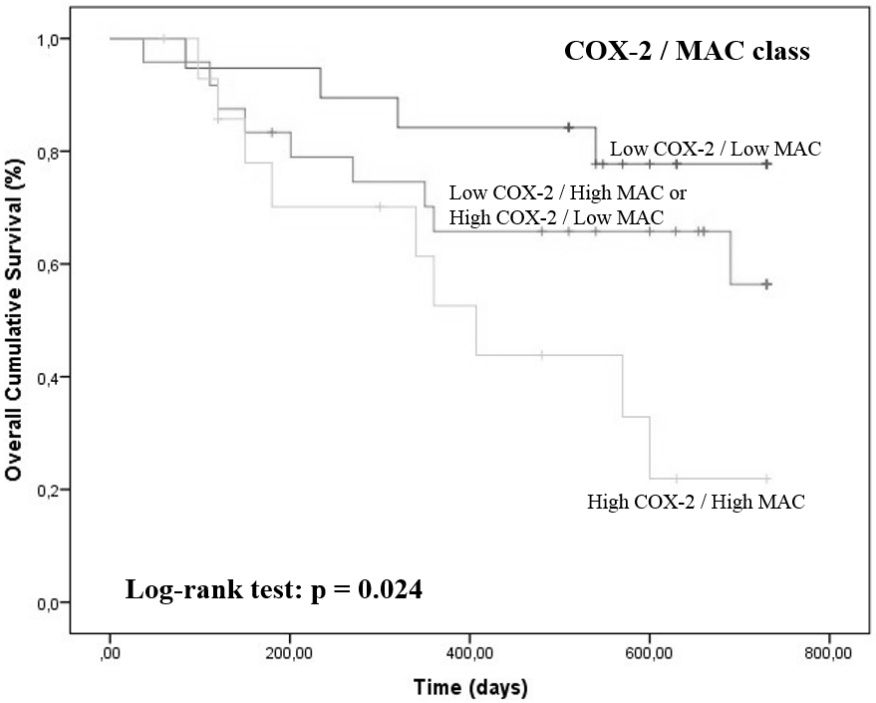

Figure 7 - Kaplan-Meier overall survival curves comparing COX-2/MAC group categories in 109 dogs with malignant mammary tumors. Female dogs with highCox-2/highMAC tumors had significantly shorter overall survival intervals.

Discussion

COX-2 can modulate tumor progression through several mechanisms: activating their receptors on tumor cells regulating cell proliferation, migration and invasion; inducing

tumor cells to secrete growth factors making a modification of normal microenvironment in order to support tumor growth and dissemination; and binding receptors on stromal cells promoting a tumor-supportive microenvironment by inducing angiogenesis and evading attack by the immune system [2, 9].

Human and dog studies report COX-2 overexpression in mammary tumors and strongly support a role for this enzyme in disease progression [34-37]. Present results demonstrate a statistically significant association of high COX-2 immunoexpression with CD31 and VEGF. Tumors with concurrent high COX-2/CD31 and high COX-2/VEGF were statistically associated with variables of tumor aggressiveness (high HGM, presence neoplastic intravascular emboli and presence of lymph node metastasis) and shorter OS of animals, both in univariate and multivariate analysis. A stable blood supply is a crucial step for tumor growth and progression. Many cells in the tumor microenvironment (tumor cells, stromal cells and immune cells) release pro-angiogenic factors stimulating endothelial cells recruitment and tubule formation [38-40]. COX-2/PGE2 pathways have been already described to be implicated in angiogenesis modulation in human breast cancer [3-5, 41, 42] and in CMT [6, 7]. However, a study in dog and cat mammary tumors presented no associations between COX-2, VEGF and MVD. [43] The present study proved that there is a direct relationship between highCOX-2/highCD31 and highCOX-2/highVEGF tumors and a lower survival rate of animals. Interestingly these groups of tumors retained the significant relationship with OS after multivariate analysis arising as a reliable independent predictors of poor prognosis in malignant CMT. PGE2 released from mammary tumor cells stimulate angiogenesis through the induction of pro-angiogenic factors: vascular endothelial growth factor (VEGF); basic fibroblast growth factor (bFGF), transforming growth factor 1 (TGF-1); platelet-derived growth factor (PDGF) and endothelin [44, 45]. Moreover COX-2/PGE2 signaling in stromal cells can induce angiogenesis by enhancing endothelial cells motility and vascular hyperpermeability [8, 46]. In addition PGE2 also stimulates immune cells to produce pro-angiogenic factors [8]. The mechanisms described above may explain the results obtained in our work.

Uncontrolled proliferation is a hallmark of malignancy in cancer and COX-2/PGE2 pathway represents an alternative mechanism by which tumors acquire growth autonomy [2]. Present work showed that high COX-2 immunoexpression revealed a statistically significant association with Ki-67 proliferation index. In addition, tumors with concurrent high COX-2 and high Ki-67 were significantly associated with unfavorable factors (high

HGM, presence of neoplastic intravascular emboli and presence of lymph node metastasis) and lower survival rate as recently observed in human breast cancer [36]. PGE2 can induce inhibition of apoptosis by increasing the expression of anti-apoptotic protein BCL-2 and decreasing the expression of pro-apoptotic protein BAX of breast cancer in humans. Furthermore PGE2 can stimulate tumor cell proliferation by upregulating aromatase production in stromal fat cells and concomitantly oestrogen production [47]. In CMT there is a study that showed no association between COX-2 and Ki-67 [43]. However a recent study in dog mammary tumor cell lines showed that selective COX-2 inhibitors suppressed cell growth by inhibition of cell proliferation [10]. The absence of other studies in CMT precludes more adequate comparisons.

Our results showed also a statistically significant association of high COX-2 immunoexpression with $CD3^+$ T-lymphocytes and MAC387 macrophages. Tumors with concurrent high COX-2/CD3 and high COX-2/MAC were statistically associated with variables of tumor aggressiveness and shorter OS. T-lymphocytes, macrophages and COX-2 are present in two intertwined phenomena: inflammation and cancer. As it has been thoroughly reported in the literature, chronic inflammation provides a specific microenvironment in which neoplastic cells find favorable circumstances to progress, growing and invading adjacent tissues [48]. Recent findings suggests that COX-2 play critical roles on T-cells and macrophages responses [19, 20, 49]. In human breast cancer PGE2 induce suppression of antigen-presenting dendritic cells, leading to a reduced activation of anti-tumor cytotoxic $CD8^+$ T-cells [21, 50] and has been reported to enhance pro-tumorigenic type 2 T-lymphocyte responses [23]. The relationship between the deregulation of PGE2 intracellular metabolism and macrophages differentiation into the M2 subtype, related to mammary carcinogenesis phenomenon, is also verified [20, 51]. In neoplastic lesions, the role of the inflammatory infiltrate is often paradoxical. The immune system is the first line of organism defense against all that is non-self, however, inflammatory cells can create, by itself, a rich microenvironment with chemical mediators that favor the development and tumor progression. So we cannot rule out the hypothesis that inflammation associated with the tumor may be, at the first stages of the disease, an attempt to reject the cancer cells, but the production of molecules such as COX-2 and VEGF can create conditions to promote tumor progression [48, 52, 53]. Accordingly with the results of present study and in light of the already known ability of COX-2 to modulate multiple aspects of the immune responses, this cancer hallmark may therefore be influenced by inappropriate expression of COX-2.

Conclusion

Present study demonstrated a link between high COX-2 immunoexpression and increased angiogenesis, proliferation and tumoral T-lymphocyte and macrophage infiltration. The tumors with concurrent high expression of COX-2 and high CD31, high VEGF, high Ki-67, high CD3 and high MAC revealed an association with parameters related with tumoral malignant phenotype (high HGM, presence of neoplastic intravascular emboli and presence of lymph node metastasis) and shorter OS of animals. Interestingly the groups high COX-2/CD31 and high COX-2/VEGF retained the significant relationship with OS after multivariate analysis arising as a reliable independent predictors of poor prognosis in malignant CMT. For the first time in a considerable series of malignant cases, it was demonstrated the concurrent COX-2 expression with variables of tumoral aggressive biological behavior, which highlight the usefulness of selective COX-2 inhibitors as a valuable therapeutic tool in malignant CMT treatment.

Table 1 - Associations of clinicopathological features with COX-2, CD31 and VEGF immunostaining.

Clinicopathological parameters	COX-2			CD31			VEGF		
	Low n	High n	p	Low n	High n	p	Low n	High n	p
Tumor size									
T1 <3cm	27	14	**0.001**	16	15		21	20	**0.002**
T2 ≥3cm and <5cm	18	17		20	15	NS	10	25	
T3 ≥5 cm	9	24		13	20		4	29	
Skin ulceration									
Absent	45	35	**0.001**	50	9	**0.004**	31	49	**0.014**
Present	35	20		9	20		4	25	
Histological type									
Tubulopapillary C.	22	26		26	22		17	31	
Solid C.	5	15		8	12		4	16	
Complex C.	21	6	**0.006**	20	7	**0.045**	13	14	**0.035**
Anaplastic C.	1	2		0	3		0	3	
Carcinosarcoma	5	6		5	6		1	10	
Tumor necrosis									
Absent	27	27	NS	31	15	**0.018**	18	28	NS
Present	19	36		28	35		17	46	
Mitotic index									
I	26	17	**0.001**	27	16	**0.004**	23	20	**<0.001**
II	22	15		24	13		9	28	
III	6	23		8	21		3	26	
Nuclear grade									
I	1	1	**<0.001**	1	1	**0.012**	0	2	**0.001**
II	39	17		38	18		27	29	
III	14	37		20	31		8	43	
Differentiation grade									
I	10	6	**<0.001**	11	5	**0.004**	9	7	**0.002**
II	31	11		29	13		18	24	
III	13	38		19	32		8	43	
HGM									
I	26	11	**<0.001**	23	14	**<0.001**	20	17	**<0.001**
II	21	10		25	6		12	19	
III	7	34		11	30		3	38	
Neoplastic intravascular emboli									
Absent	43	31	**0.009**	48	26	**0.01**	26	48	NS
Present	11	24		11	24		9	26	
Lymph node metastasis									
Absent	44	28	**0.002**	50	22	**<0.001**	31	41	**0.001**
Present	10	27		9	28		4	33	

n, number of samples; p, statistical significance; C, carcinoma; NS, not significant.

Table 2 - Associations of clinicopathological features with Ki-67, $CD3^{+}$T-lymphocytes and macrophages immunostaining.

Clinicopathological parameters	Ki-67			CD3			MAC		
	Low n	High n	p	Low n	High n	P	Low n	High n	p
Tumor size									
T1 <3cm	27	14		29	12	NS	30	11	NS
T2 ≥3cm and <5cm	20	15	NS	22	13		20	15	
T3 ≥5 cm	15	18		15	18		18	15	
Skin ulceration									
Absent	46	34	NS	54	26	**0.014**	53	15	NS
Present	16	13		12	17		15	14	
Histological type									
Tubulopapillary C.	29	19		32	16		34	14	
Solid C.	7	13		8	12		9	11	NS
Complex C.	17	10	NS	18	9	NS	17	10	
Anaplastic C.	2	1		1	2		2	1	
Carcinosarcoma	7	4		7	4		6	5	
Tumor necrosis									
Absent	28	18	NS	36	10	**0.001**	26	20	NS
Present	34	29		30	33		42	21	
Mitotic index									
I	33	10	**0.002**	29	14	NS	32	11	NS
II	18	19		24	13		20	17	
III	11	18		13	16		16	13	
Nuclear grade									
I	0	2	**<0.001**	2	0	**0.048**	2	0	NS
II	42	14		39	17		38	18	
III	20	31		25	26		28	23	
Differentiation grade									
I	14	2	**0.005**	12	4	**0.001**	12	4	**0.026**
II	26	16		33	9		31	11	
III	22	29		21	30		25	26	
HGM									
I	29	8	**<0.001**	27	10	**0.002**	31	6	**0.004**
II	19	12		23	8		16	15	
III	14	27		16	25		21	20	
Neoplastic intravascular emboli									
Absent	48	26	**0.014**	54	20	**<0.001**	46	22	NS
Present	14	21		12	23		22	13	
Lymph node metastasis									
Absent	48	24	**0.004**	55	17	**<0.001**	51	21	**0.011**
Present	14	23		11	26		17	20	

n, number of samples; p, statistical significance; C, carcinoma; NS, not significant.

Table 3 - Relationship of COX-2/CD31; COX-2/VEGF; COX-2/Ki-67; COX-2/CD3 and COX-2/MAC groups with clinicopathological variables of tumor aggressiveness.

Molecular markers		Variables of tumor aggressiveness								
		HGM			Neoplastic intravascular emboli			Lymph node metastasis		
		Low (I/II)	High (III)	p	Absent	Present	p	Absent	Present	p
COX-2/CD31	LowCOX-2/LowCD31	36	3	**<0.001**	35	4	**<0.001**	34	5	**<0.001**
	LowCOX-2/HighCD31 or HighCOX-2/LowCD31	23	12		21	14		26	9	
	HighCOX-2/HighCD31	9	26		18	17		12	23	
COX-2/VEGF	LowCOX-2/LowVEGF	24	2	**<0.001**	18	7	**<0.001**	22	3	**<0.001**
	LowCOX-2/HighVEGF or HighCOX-2/LowVEGF	32	7		33	6		31	8	
	HighCOX-2/HighVEGF	13	31		23	22		19	26	
COX-2/Ki-67	LowCOX-2/LowKi-67	34	2	**<0.001**	29	7	**0.001**	32	4	**<0.001**
	LowCOX-2/HighKi-67 or HighCOX-2/LowKi-67	26	16		32	10		27	15	
	HighCOX-2/HighKi-67	8	23		13	18		13	18	

Table 3 – Continued

Molecular markers		Variables of tumor aggressiveness								
		HGM			Neoplastic intravascular emboli			Lymph node metastasis		
		Low (I/II)	High (III)	p	Absent	Present	p	Absent	Present	p
COX-2/CD3	LowCOX-2/LowCD3	39	4	**<0.001**	37	6	**<0.001**	38	5	**<0.001**
	LowCOX-2/HighCD3 or HighCOX-2/LowCD3	18	15		22	11		22	11	
	HighCOX-2/HighCD3	11	22		15	18		12	21	
COX-2/MAC	LowCOX-2/LowMAC	33	4	**<0.001**	27	10	NS	32	6	**<0.001**
	LowCOX-2/HighMAC or HighCOX-2/LowMAC	26	19		33	12		30	15	
	HighCOX-2/HighMAC	9	18		14	13		10	17	

n, number of samples; p, statistical significance; NS, not significant.

Table 4 - Association between considered molecular markers groups and overall survival.

Molecular markers groups		n	Overall Survival Univariate (Mean values)	p	Overall Survival Multivariate* (Hazard ratio)	p
COX-2	LowCOX-2	51	648.447	**<0.001**	-	NS
	HighCOX-2	58	463.755			
COX-2/CD31	LowCOX-2/LowCD31	39	655.960	**0.008**	**0.275** (CI: 0.051 - 1.481)	**0.04**
	LowCOX-2/HighCD31 or HighCOX-2/LowCD31	35	560.925			
	HighCOX-2/HighCD31	35	439.000			
COX-2/VEGF	LowCOX-2/LowVEGF	25	693.639	**<0.001**	**7.049** (CI: 0.570 - 27.446)	**0.012**
	LowCOX-2/HighVEGF or HighCOX-2/LowVEGF	39	634.524			
	HighCOX-2/HighVEGF	45	416.752			
COX-2/Ki-67	LowCOX-2/LowKi-67	36	652.379	**<0.001**	-	NS
	LowCOX-2/HighKi-67 or HighCOX-2/LowKi-67	42	553.929			
	HighCOX-2/HighKi-67	31	344.363			
COX-2/CD3	LowCOX-2/LowCD3	43	660.132	**0.002**	-	NS
	LowCOX-2/HighCD3 or HighCOX-2/LowCD3	33	529.506			
	HighCOX-2/HighCD3	33	452.894			
COX-2/MAC	LowCOX-2/LowMAC	37	636.008	**0.024**	-	NS
	LowCOX-2/HighMAC or HighCOX-2/LowMAC	45	545.719			
	HighCOX-2/HighMAC	27	426.518			

n, number of samples; p, statistical significance; NS, not significant; CI, confidence interval; *Multivariate Cox Proportional Hazard Analysis (Cox Regression) of overall survival (OS) in dogs with malignant mammary tumors in a prospective study with 2 years of follow-up.

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III.2 Positive Interplay between $CD3^+$ T-lymphocytes and Concurrent COX-2/EGFR expression in Canine Malignant Mammary Tumors

Abstract

The ability of tumors to evade the immune system is one of cancer hallmarks. In breast cancer, it was demonstrated that the cyclooxygenase-2^+/epidermal growth factor receptor$^+$ (COX-2^+/$EGFR^+$) status might influence tumor microenvironment allowing the escape of cancer cells to the immune system. This topic is unknown in canine mammary tumors (CMT). Therefore, the potential relationship between $CD3^+$ T-lymphocytes and concurrent COX-2/EGFR expression was investigated. Formalin-fixed paraffin-embedded malignant CMT samples (n = 63) were submitted to immunohistochemical staining to detect CD3, COX-2 and EGFR. Tumoral $CD3^+$ T-lymphocytes were significantly associated with tubular differentiation grade ($p = 0.006$), tumor necrosis ($p = 0.025$), histological grade of malignancy ($p = 0.027$) and presence of lymph node metastasis ($p = 0.009$). A correlation between COX-2 and EGFR was observed ($r = 0.741$, $p < 0.001$). The COX-2^+/$EGFR^+$ group was associated with tumor size ($p = 0.002$), mitotic index ($p = 0.019$), histological grade of malignancy ($p = 0.035$) and presence of lymph node metastasis ($p = 0.041$). $CD3^+$ T-lymphocytes and COX-2/EGFR group were significantly associated ($p = 0.025$) and positively correlated ($r = 0.399$; $p = 0.003$). The present results suggest that the COX-2^+/$EGFR^+$ status may be part of a strategy adopted by tumor cells to evade the cytotoxic tumor-specific immune responses.

Introduction

Mammary tumorigenesis involves a complex and intricate interplay between tumor and stromal cells. The supportive tumoral microenvironment (fibroblasts, adipocytes and immune cells) surrounds primary tumor cells and appears to have a critical role in tumor progression towards malignancy [1-3].

In human breast cancer [4, 5] and in canine mammary tumors (CMT) [6-8], several studies attributed an important role to $CD3^+$ T-lymphocytes, as well as cytokines produced by them. The evidences suggest that T-lymphocytes might cooperate with the tumor cells favoring tumor development and progression [3, 4, 6-9].

Cyclooxigenase-2 (COX-2) over-expression has been related with tumor aggressiveness in human breast cancer [10, 11] and in CMT [12-16] and there has been a great interest in a better understanding of the signaling pathways that underlying COX-2 expression. Several appointed mechanisms, which include deregulated growth factor signaling and oncogene activation, have been reported [17]. Examples of these mechanisms comprise activation of the Wnt pathway [18, 19] and the Ras-MAPK pathway [20] signaling *via* growth factor receptors, including epidermal growth factor receptor (EGFR) [21]. COX-2 expression and prostaglandin E2 (PGE2) production have been shown to up-regulate the EGFR, PI3K and ERK1/2 signaling, thereby inducing angiogenesis, cell proliferation and invasion [17, 22]. COX-2 and EGFR molecules demonstrated to share some functions in common signaling pathways in several stages of mammary carcinogenesis by mediating pleiotropic carcinogenic processes both in humans and dogs [16, 23] .

In human breast cancer, COX-2 has influence on tumor and stromal cells interplay and COX-2-derived PGE2 contributes to matrix remodeling, modulates multiple aspects of the immune responses and supports the suppressed immune surveillance [17, 24-26]. PGE2 has the ability to regulate the immune system modulating the functions of different cell populations, including T-lymphocytes [1, 26, 27], and has been reported to enhance pro-tumorigenic type 2 lymphocytes and myeloid cell functions promoting angiogenesis and supporting tumor growth [1, 27, 28].

COX-2 modulates and suppresses immune function in human breast cancer [28] and up-regulates the EGFR activity by a positive feedback loop in human and dog mammary tumors [16, 23], which raises the hypothesis that the ability of tumor cells to evade the immune system may, therefore, be influenced by inappropriate concurrent expression of COX-2/EGFR.

COX-2 and EGFR are promising therapeutic targets; therefore, the relationship between $CD3^+$ T-lymphocytes and concurrent COX-2/EGFR expression was investigated in this study, since the better understanding of these molecular interplays may be useful in developing clinically effective immunotherapeutic approaches.

Materials and Methods

Tissue samples

In this study, 63 malignant canine mammary tumors were included. Samples were surgically excised with curative intent from 63 dogs that expressed natural tumor occurrence. All specimens were fixed in 10% buffered formalin, paraffin-embedded and 4 μm sections were sequentially cut from each block, following routine methods. One section was stained with hematoxylin and eosin for histopathological diagnosis and subsequent sections were used for immunohistochemical studies. The histopathological diagnosis of tumors was performed by the WHO classification for CMT [29] by two independent pathologists (IP and JP). The clinicopathological characteristics, evaluated in each sample, were tumor size (T1 <3 cm; T2 ≥3 and <5 cm; T3 ≥5 cm), skin ulceration, presence of necrosis, mitotic index, nuclear grade, tubular differentiation grade, histological grade of malignancy and regional lymph node metastasis. Mitotic index was assessed in 10 high-power fields (HPFs) (×400) and classified in 3 grades according to the recommended guidelines [30]. Nuclear grade, tubular differentiation grade and histological grade of malignancy were also evaluated according to the recent recommendations for CMT grading [30].

Immunohistochemical analysis

Immunohistochemistry (IHC) was performed using the streptavidin-biotin-peroxidase complex method with the Ultra Vision Detection System kit (Lab Vision Corporation, Fremont, CA, USA) for CD3 and COX-2, while for EGFR was used a polymeric labeling methodology (Novolink Polymer Detection System; Novocastra, Newcastle, UK) following the manufacturer's instructions. Sections were dewaxed in xylene and rehydrated through graded alcohols. For CD3 and COX-2, antigen retrieval was executed by microwave treatment for 3x5 min at 750W in 0.01 M citrate buffer, pH = 6.0, followed by cooling at room temperature for 20 min. For EGFR, antigen retrieval was carried out by enzyme digestion: sections were incubated with 0.4% pepsin (Dako, Glostrup,

Denmark) in HCl 0.01 N solution (pH = 2) for 30 min at 37 ºC. All sections were incubated with specific antibodies: CD3 (polyclonal antibody; at 1:50 dilution; Dako, Glostrup, Denmark) for 2 h at room temperature; COX-2 (Clone SP21; at 1:40 dilution, Transduction Laboratories, Lexington, Kentucky, USA) for 24 h at 4 ºC; EGFR (clone 31G7; at 1:100 dilution; Invitrogen, Paisley, Scotland, UK) for 45 min at room temperature. The antibody reaction products were observed with the cromagen 3, 3'-diaminobenzidine tetrachloride (DAB) at 0.05% with 0.01% H_2O_2 (30%). After a final washing in distilled water, the sections were counterstained with hematoxylin, dehydrated, cleared and mounted. The primary antibody was replaced with phosphate-buffered saline (PBS) for negative controls; this study also included adequate positive controls. Sections of canine lymph nodes were used as positive control for CD3. For COX-2, macula densa of young dog kidney was used, while the epidermis was used as internal positive control for EGFR.

Quantification of immunolabelling

The immunolabelling quantification was done by two independent observers (MIC and FLQ). To evaluate intratumoral CD3 expression, the three regions in the tumor with the most intense and homogeneous positivity were selected. In these regions, all labeled cells were counted, evaluating a total of 10 high power fields (HPFs) (×400) following a quantitative method used previously by our team [6]. To evaluate COX-2 and EGFR expression, a previously applied semiquantitative method [16, 31] adapted from Ceccarelli and colleagues [32] was used. This method was based on the estimates of the percentage of positive cells (immunolabelling extension) and the staining intensity.

Statistical analysis

The statistical software SPSS (Statistical Package for the Social Sciences), version 19.0 (IBM SPSS Statistics), was used for statistical analysis. The Chi-square test was used to study the categorical variables. Analysis of variance (ANOVA) was used for analyzing continuous variables. The Pearson's correlation test was performed in order to verify the presence of correlation between values of CD3, COX-2 and EGFR. All values were expressed as means ± standard error. In all statistical comparisons, $p < 0.05$ was regarded as significant.

Results

Tumors

The present study comprised 63 malignant canine mammary tumors, including 3 *in situ* carcinomas (4.8%), 10 complex carcinomas (15.9%), 32 tubulopapillary carcinomas (50.7%), 8 solid carcinomas (12.7%), 7 carcinosarcomas (11.1%) and 3 anaplastic carcinomas (4.8%). Nineteen malignant tumors were grade I, 20 grade II and 24 grade III. Within the 48 cases, where lymph nodes were available, 20 (41.67%) had metastasis.

$CD3^+$ T-lymphocytes, COX-2 and EGFR immunostaining

$CD3^+$ T-lymphocyte were present in all samples ranging from 16 to 356 lymphocytes in 10 HPFs. CD3 immunostaining was observed in the cytoplasm and/or in the cytoplasmatic membrane of T-lymphocytes in a diffuse and homogeneous pattern. T-lymphocytes tend to contact closely with neoplastic cells and the diffuse inflammation emerged as the predominant pattern of infiltration. Sometimes, although less frequent, T-lymphocytes were also accumulated in perilobular and perivascular clusters.
Immunostaining for COX-2 and EGFR was also performed in all cases. The immunoreactivity for COX-2 was observed in the cytoplasm, nuclear membrane and cytoplasmatic membrane, in a diffuse and homogeneous manner. Thirty one of the 63 cases demonstrated high immunoreactivity for COX-2. The immunoreactivity for EGFR was observed at the cytoplasmatic membrane and within the cytoplasm of the neoplastic cells, in a diffuse pattern. Thirty nine of the 63 cases showed high immunoreactivity for EGFR.

Relationship of $CD3^+$ T-lymphocytes with clinicopathological variables

The present results demonstrated an association between tumoral $CD3^+$ T-lymphocytes and the tubular differentiation grade ($p = 0.006$) showing that poorly differentiated tumors (with less tubular formation) demonstrated increased $CD3^+$ infiltration. An association was also observed between increased $CD3^+$ infiltration and presence of tumor necrosis ($p = 0.025$), high histological grade of malignancy ($p = 0.027$) and presence of lymph node metastasis ($p = 0.009$). All results are summarized in **Table 5**.

Table 5 - Relationship between tumoral $CD3^+$ T-lymphocytes and clinicopathological parameters.

	Tumoral CD3			
Clinicopathological parameters	**n**	**Mean**	**SE**	**p**
Tumor size				
T1 <3cm	20	92.81	22.69	
T2 ≥3cm and <5cm	19	59.80	6.87	NS
T3 ≥5cm	24	122.20	23.80	
Skin ulceration				
Absent	46	89.47	13.19	NS
Present	17	120.50	33.20	
Histological type				
In situ C.	3	35.00	10.00	
Tubulopapillary C.	32	103.72	10.00	
Solid C.	8	164.14	42.72	NS
Complex C.	10	77.56	26.81	
Anaplastic C.	3	27.00	-	
Carcinosarcoma	7	52.00	9.24	
Tumour necrosis				
Absent	36	72.83	11.15	**0.025**
Present	27	128.47	23.77	
Mitotic index				
I	28	67.62	10.37	
II	16	114.58	32.55	NS
III	19	139.40	30.62	
Nuclear grade				
I	7	48.33	7.69	
II	28	105.62	19.74	NS
III	28	100.37	20.11	
Differentiation grade				
I	14	58.40^a	16.92	
II	24	69.00^a	9.91	**0.006**
III	25	148.8^b	26.62	
HGM				
I	19	68.78^a	10.67	**0.027**
II	20	92.58^{ab}	29.31	
III	24	149.7^b	29.82	
Lymph node metastasis				
Absent	28	72.74	11.64	**0.009**
Present	20	146.71	30.27	

n, number of samples; se, standard error; p, statistical significance; C, carcinoma; NS, not significant. Mean values with different superscript letters denote statistically significant differences on each item considered - Tukey Post Hoc Test

Correlation between COX-2 and EGFR and relationship of COX-2/EGFR groups with clinicopathological variables.

A positive and statistically significant correlation between COX-2 and EGFR immunoreactivity was observed ($r = 0.741$, $p < 0.001$) and there were not any tumors with elevated COX-2 and low EGFR expression. Thirty one of the 63 tumors (49.2%) with high COX-2 immunoreactivity had also high EGFR immunostaining. In this study, the COX-2/EGFR groups were considered: low COX-2/low EGFR (n = 24); low COX-2/high EGFR (n = 8); high COX-2/high EGFR (n = 31). The COX-2/EGFR groups were statistically significantly associated with tumor size ($p = 0.002$), mitotic index ($p = 0.019$), histological grade of malignancy ($p = 0.035$) and presence of lymph node metastasis ($p = 0.041$). More information is provided in **Table 6**.

$CD3^+$ T-lymphocytes and COX-2/EGFR groups associations

A significant association between $CD3^+$ T-lymphocytes and COX-2/EGFR groups was observed ($p = 0.025$). The group with high COX-2 and high EGFR demonstrated higher counts of tumoral $CD3^+$ T-lymphocytes (**Figure 8**).

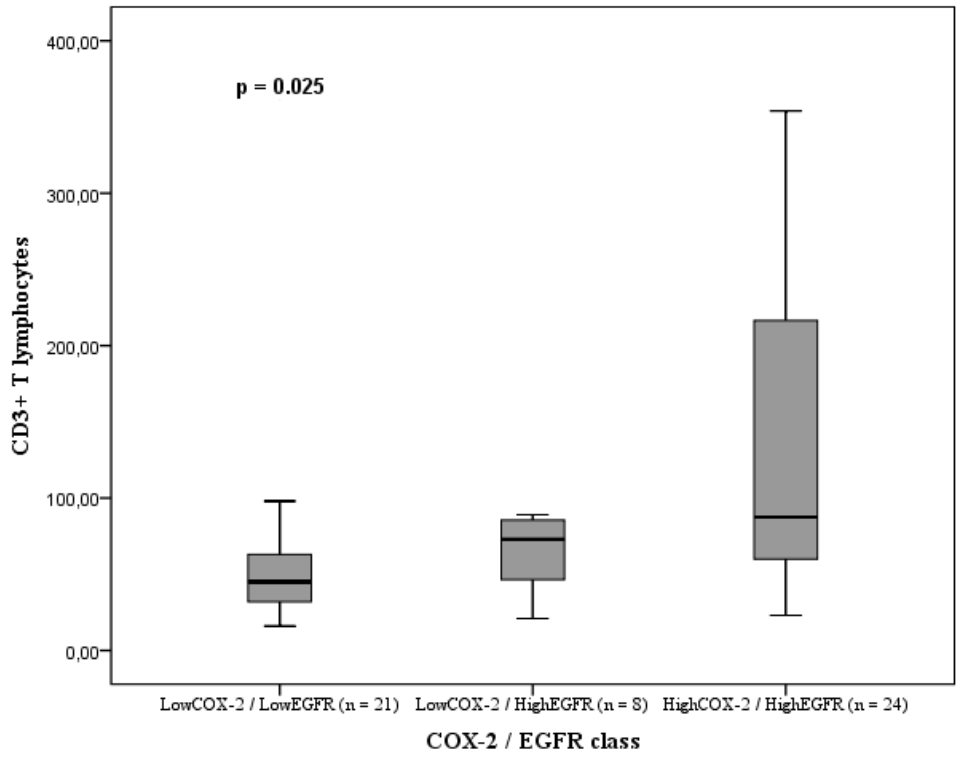

Figure 8 - Association of $CD3^+$ T-lymphocytes and COX-2/EGFR groups in malignant canine mammary tumors.

Correlation between CD3$^+$ T-lymphocytes and COX-2/EGFR groups

In this study, a positive and statistically significant correlation between CD3$^+$ T-lymphocytes and COX-2/EGFR groups was observed (r = 0.399; p = 0.003).

Table 6 - Relationship of COX-2/EGFR groups with clinicopathological variables.

Clinicopathological parameters	Low COX-2/low EGFR n	Low COX-2/high EGFR n	High COX-2/high EGFR n	p
Tumor size				
T1 <3cm	13	2	5	
T2 ≥3cm and <5cm	8	3	8	**0.002**
T3 >5cm	3	3	18	
Skin ulceration				
Absent	17	7	22	NS
Present	7	1	9	
Histological type				
In situ C.	1	0	2	
Tubulopapillary C.	14	3	15	
Solid C.	1	0	7	NS
Complex C.	5	1	4	
Anaplastic C.	0	3	0	
Carcinosarcoma	3	1	3	
Tumor necrosis				
Absent	17	6	13	NS
Present	7	2	18	
Mitotic index				
I	16	3	9	**0.019**
II	4	2	10	
III	4	3	12	
Nuclear grade				
I	5	0	2	NS
II	10	3	15	
III	9	5	14	
Differentiation grade				
I	6	1	7	NS
II	11	4	9	
III	7	3	15	
HGM				
I	10	2	7	**0.035**
II	8	4	8	
III	6	2	16	
Lymph node metastasis				
Absent	11	4	13	**0.041**
Present	5	3	12	

n, number of samples; p, statistical significance; C, carcinoma; NS, not significant.

Discussion

Tumor-associated T-lymphocyte responses can be generalized to type 1 and type 2 in which Th1 lymphocytes limit tumor development and Th2 lymphocytes favor immune escape and disease progression. Both human and dog cancer patients seem to demonstrate a lymphocyte dysfunction characterized by an imbalance of the normal ratio of Th1/Th2 cells [1, 9, 33-35].

In human breast cancer, recent findings suggests that COX-2 and COX-2-derived products, particularly PGE2, act in tumor cells *via* classical cancer signaling pathways promoting tumorigenesis and playing critical roles in T-cell responses, suppressing cytotoxic T cell actions against the tumor [26]. PGE2 has been also reported to enhance pro-tumorigenic type 2 lymphocytes [28] and to up-regulate the EGFR *via* by a positive feedback loop [23]. COX-2/EGFR up-regulated pathways have been described as a major determinant for breast cancer progression and metastasis largely due to the ability to regulate and suppress the cytotoxic responses of the immune system [17, 26, 36, 37]. In CMT, this topic remains unclear and, to the best of our knowledge, this is the first study that investigates the relationship between $CD3^+$ T-lymphocytes and concurrent COX-2/EGFR immunoexpression.

The present study revealed a relationship between high tumoral $CD3^+$ T-lymphocytes and presence of tumor necrosis, high differentiation grade, high histological grade of malignancy and presence of lymph node metastasis. These results suggest an association of $CD3^+$ T-lymphocytes and more aggressive tumor phenotypes reflecting the involvement of T-lymphocytes in canine mammary malignancy. Our results are in agreement with previous published works in CMT [6-8, 38, 39] and suggest that the immune system may release factors that contribute to tumor survival, growth and invasion. Tumor cells might, thus, use a multitude of mechanisms to escape from cytotoxic T-cell actions and additionally be subject to the polarity of the pro-tumorigenic Th2 cell responses, which also work favoring tumor protection [9].

Concerning the concurrent COX-2/EGFR immunoexpression, the present results revealed a positive and statistically significant correlation between the two markers. Tumors with high COX-2 and EGFR immunoexpression were statistically associated with larger tumor size, high mitotic index, high histological grade of malignancy and presence of lymph node metastases. These results were already observed by our team in a small set of tumors [16] and are in agreement with studies in human cancer confirming the common aspects

of the interactive signaling pathways between COX-2 and EGFR in both species [16, 37]. Considerable evidence indicates that COX-2–derived PGE2 can activate EGFR signaling and, thereby, stimulate tumor cell proliferation, invasion and metastasis [23]. Interestingly, in the current study, the concurrent COX-2/EGFR positive expression was significantly associated with higher tumoral $CD3^+$ T-lymphocytes. Furthermore, a positive and statistically significant correlation was observed. According to the present results, tumoral $CD3^+$ T-lymphocytes may be influenced by inappropriate expression of COX-2/EGFR. COX-2 over-expression and the resulting increase in PGE2 levels could induce over-expression of EGFR, possibly representing a strategy adopted by tumors that contributes to the evasion of tumor-specific immune response. PGE2 induces suppression of antigen-presenting dendritic cells leading to a reduced activation of anti-tumor cytotoxic $CD8^+$ T-cells [24, 40]. PGE2 also has inhibitory effects on T-cell apoptosis and decreases production of interferon gamma (IFNγ) and interleukin-2 (IL-2) [27, 41, 42]. The cellular effects of PGE2 are mediated through four prostaglandin E receptors, EP1, EP2, EP3 and EP4 that are associated to different intracellular signaling pathways [43]. Proliferation of Th1 cells is inhibited through EP2 [44]. The EP2 and, maybe, the EP4 receptors mediated the suppressive effects of PGE2 on cytotoxic T-cells [45].

The results of our work suggest that similar mechanisms may be present in CMT. The interaction between COX-2/EGFR and $CD3^+$ T-lymphocytes highlights the molecular connection between cancer therapy and cancer prevention and the growing importance of molecular targeted approaches. However, the mechanisms through which COX-2/EGFR influence the T-lymphocyte functions are still poorly defined emphasizing the need for additional studies in this area.

Conclusion

The findings of our study support future investigations concerning the better understanding of the crosstalk between COX-2/EGFR signaling pathways and $CD3^+$ T-lymphocytes. The significant correlation of COX-2/EGFR with $CD3^+$ T-lymphocytes and the relationship of the molecular markers with more aggressive tumor phenotypes justify the need to pursue further studies considering clinically effective immunotherapeutic approaches against CMT.

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III.3 Intratumoral $CD3^+$ T-lymphocytes immunoexpression and its association with c-kit, Angiogenesis and Overall Survival in Malignant Canine Mammary Tumors

Abstract

The c-kit signaling pathways have been described as being involved in migration and differentiation of T-cells into tumor site. This topic is unknown in canine mammary tumors (CMT). Therefore 80 malignant samples were submitted to immunohistochemical detection of CD3, c-kit, VEGF and CD31, together with clinicopathological parameters of tumor aggressiveness. $CD3^+$ T-cells and c-kit overexpression revealed a positive correlation with VEGF ($r = 0.503$, $p < 0.001$; $r = 0.284$, $p = 0.023$ for CD3 and c-kit respectively) and CD31 ($r = 0.654$, $p < 0.001$; $r = 0.365$, $p = 0.003$ for CD3 and c-kit respectively). A significant association ($p = 0.039$) and a positive correlation ($r = 0.263$, $p = 0.039$) between CD3 and c-kit was also observed. High CD3/VEGF, high c-kit/VEGF and high CD3/c-kit tumors were associated with elevated grade of malignancy ($p < 0.001$ for all groups), presence of intravascular emboli ($p < 0.001$ for CD3/VEGF and CD3/c-kit; $p = 0.002$ for c-kit/VEGF) and presence of lymph node metastasis ($p < 0.001$ for all groups). Tumors with high CD3/VEGF ($p = 0.006$), c-kit/VEGF ($p < 0.001$) and CD3/c-kit ($p = 0.002$) were associated with poor prognosis. Interestingly high c-kit/VEGF tumors retained their significance by multivariate analysis arising as independent prognostic factor. Present results suggest that T-lymphocytes may share common signaling pathways with c-kit and VEGF in CMT progression and may contribute to increased angiogenesis, aggressiveness and poor prognosis in these tumors.

Introduction

Tumor-associated inflammatory response is a highly coordinated event involving several key players and have the effect of enhancing mammary tumorigenesis, helping emerging neoplasias to acquire hallmark capabilities [1, 2]. Chronic inflammation contribute to activation of important facilitating programs by providing active molecules to the tumor microenvironment, including growth and survival factors; pro-angiogenic factors as VEGF, and extracellular matrix-remodeling enzymes that allow angiogenesis, invasion, and metastasis [3-5]. Additionally, inflammatory cells, can contribute to mutagenic transformation of cancer cells, accelerating their genetic evolution toward states of higher malignancy [6, 7]. However is still incompletely understood how the chronic inflammation in the mammary tumor microenvironment is coordinated by inflammatory cells themselves.

Recent studies, in human breast cancer [8, 9] and canine mammary tumors (CMT) [10-12], highlight T-lymphocytes as an important regulator of inflammation and their accumulation in tumor sites has also been well documented [8, 10]. T-cells migration to tumor site and the following activation may be the essential requirement for their local promoting effect [2, 13, 14]. Nevertheless, how T-lymphocytes are recruited into the tumor site and whether they can remodel the tumor microenvironment, is a key question that remains unclear. In several human tumors, including breast cancer, the c-kit signaling has been described as being implicated in differentiation and migration of T-cells in tumor sites [15, 16]. The deregulation of c-kit induce the activation of several signaling pathways that can result in chronic inflammation with immune balance from activation to tolerance [15, 17, 18] which may be a deleterious immune condition in a variety of diseases, including cancer and may be implicated in mammary tumorigenesis.

Nevertheless all the evidence, overexpression of c-kit still represents a highly controversial subject in breast cancer. Several studies propose that the loss of c-kit expression has been associated with tumor progress, whereas other reports indicate overexpression of c-kit related to increase angiogenesis and tumor development [19-24]. In CMT few studies have examined the expression of c-kit suggesting that c-kit mutation and activation may be involved in the pathogenesis of these tumors [25-27]. However, to our knowledge, there are no studies in human breast cancer or in CMT that focus on relationship between T-lymphocytes, c-kit expression and tumoral angiogenesis and aggressiveness. Present study aims to explore the possible common signaling/regulatory

pathways between c-kit and T-lymphocyte responses in CMT which may potentiate applications in immunological therapeutic strategies and provide new insights into the role of c-kit in inflammation, immunosuppression and tumor progression.

Materials and Methods

Mammary tumors and clinicopathological variables

This study included 80 malignant CMT excised, with curative intent, from dogs received for diagnosis and treatment. Samples were fixed in 10% formalin, processed using an automatic tissue microprocessor, and paraffin embedded. Paraffin wax blocks were cut into 4 µm sections using a microtome, mounted on glass slides, and stained with hematoxylin–eosin for diagnostic purposes, according to the WHO criteria for CMT [28]. Tumors were graded in accordance with the method proposed by Goldschmidt and colleagues [29]. The following clinicopathological parameters were evaluated in each sample: tumor size (T1 <3 cm; T2 ≥3 and <5 cm; T3 ≥5 cm), skin ulceration, tumor necrosis, mitotic index, nuclear grade, differentiation grade, histological grade of malignancy, neoplastic intravascular emboli and regional lymph node involvement.

Immunohistochemical technique

Immunoexpression of CD3, c-kit, CD31 and VEGF was carried out using the streptavidin–biotin–peroxidase complex method, with a commercial detection system (Ultra Vision Detection System; Lab Vision Corporation, Fremont, California, USA) following the manufacturer's instructions. All slides were subjected to microwave antigen retrieval before immunolabelling, with a citrate buffer, for 3x5 min at 750W.

As the primary specific antibody we used: CD3 (polyclonal antibody, Dako, Glostrup, Denmark, 1:50 dilution), during 2 hours at room temperature; c-kit (a polyclonal rabbit anti-human c-kit protein; Dako, Carpinteria, California, USA, 1:100 dilution), CD31 (Clone JC70A, Dako, Glostrup, Denmark; 1:20 dilution), VEGF (Clone JH121, Thermo Scientific, Waltham, MA USA; 1:100 dilution), in overnight incubation at 4ºC. Immunoreaction was visualized by slide incubation with 0.05% 3.30-diaminobenzidine tetrahydrochloride (DAB) with 0.01% hydrogen peroxide (H_2O_2) as the final substrate, for 10 min. After a final washing in distilled water, the sections were counterstained with hematoxylin, dehydrated, cleared, and mounted with Entellan resin (Merck KGaA, Darmstadt, Germany). As a positive control, we used: sections of canine lymph nodes

for CD3; sections of cutaneous grade III mast cell tumors with known positivity to c-kit; dog angiosarcoma for CD31 and liver section for VEGF. As a negative control, the primary antibody was replaced by PBS.

Evaluation of immunoreactivity

The assessment of CD3, CD31 and VEGF immunolabelling was based on methods previously used in CMT by our team [10, 30-32].

The c-kit immunoreactivity was evaluated in accordance to parameters already described in canine cutaneous melanocytic tumors [33] and in CMT [25] by determining the percentage of positively labeled cells and the labeling intensity. The percentage of positively labeled cells (extension) was scored as: negative (0%), focal (1–19%), intermediate (20–49%), or diffuse (>50%). The labeling intensity was scored as: negative (–), weak (+), moderate (++), or strong (+++). Subsequently, the two scores were combined (sum) and a final score was obtained: Low immunoreactivity (score values ≤ 4) and High immunoreactivity (score values 5-6). The labeling location was recorded as cytoplasmic, membranar or both.

Clinical follow-up

Dogs were clinically examined by veterinarians every 90 - 120 days after surgical treatment for a minimum of 730 days. Follow-up included a radiological evaluation of the thorax and an abdominal ultrasound. For the animals that died within the 730 days period, overall survival (OS) was considered the number of days between surgery and death, whilst for the dogs who survived > 730 days, the OS was the number of days from surgery to the last clinical examination. Follow-up was carried out for a mean period of 472 days (minimum 37, maximum 730 days).

Statistical analysis

The Chi-square test was used to study the categorical variables. Analysis of variance (ANOVA) was used for analyzing continuous variables. The Pearson's correlation test was performed in order to verify the presence of correlation between values of CD3, c-kit, VEGF and CD31. Influence on survival was established using the Log Rank test and cases were grouped in low or high according to expression. Cox proportional hazards model for multivariate analysis was also implemented. Analysis were performed using

the statistical software SPSS (Statistical Package for the Social Sciences) version 19.0 and a conventional 5% level was used to define statistical significance.

Results

The present study comprised 38 tubulopapillary carcinomas, 14 complex carcinomas, 12 solid carcinomas, 3 anaplastic carcinomas and 13 carcinosarcomas. Twenty malignant tumors were grade I, 20 grade II and 40 grade III. Twenty-five tumors (31.25%) had neoplastic intravascular emboli and 31 cases (38.75%) had lymph node metastasis.

Expression of CD3$^+$ T-lymphocytes, c-kit, VEGF and CD31 in CMT

The CD3 immunostaining was observed in the cytoplasm or/and in the cytoplasmatic membrane of T-lymphocytes and the diffuse inflammation was the predominant pattern of infiltration, as previous described [10]. The mean number (±SD) of total intratumoral CD3$^+$ T-lymphocytes was 116.6 (±84.3), minimum 26 and maximum 364. The c-kit immunoexpression was mainly cytoplasmic (92.5%, n = 74), with a few cases having simultaneous cytoplasmic and membranar labelling (7.5%, n = 6; **Figure 9**). In intratumoral area the labelled cells were mostly epithelial, however was observed also immunoreactivity in malignant myoepithelial cells and sarcoma cells of carcinosarcomas (**Figure 10**).

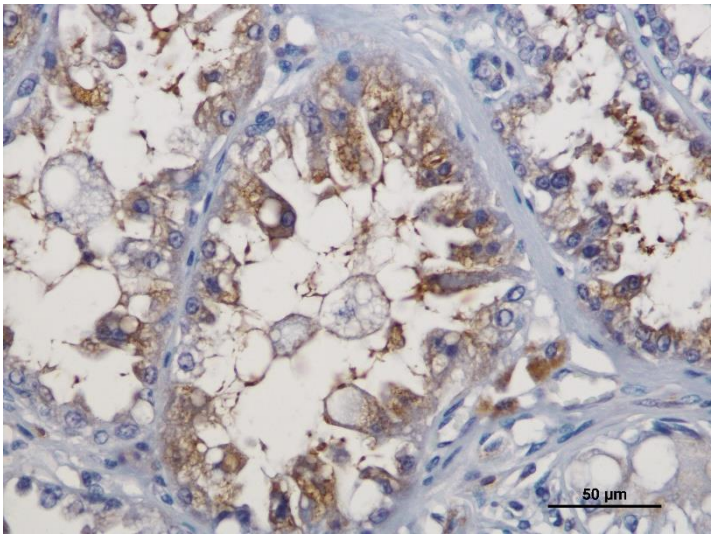

Figure 9 - Immunoreactivity for c-kit in tubulopapillary carcinoma; note the simultaneous cytoplasmic and membranous staining; bar =50 μm.

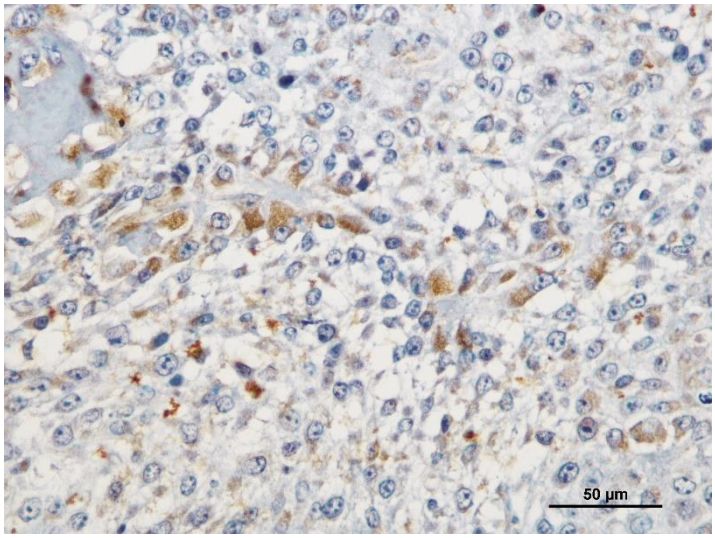

Figure 10 - Immunoreactivity for c-kit in carcinosarcoma; bar =50 μm.

In adnexal non-tumoral mammary gland c-kit expression was observed in alveolar and ductal mammary epithelium in smaller proportions relative to the tumor (**Figure 11**).

For immunolabelling extension, most tumors showed a diffuse immunolabelling (60%, n

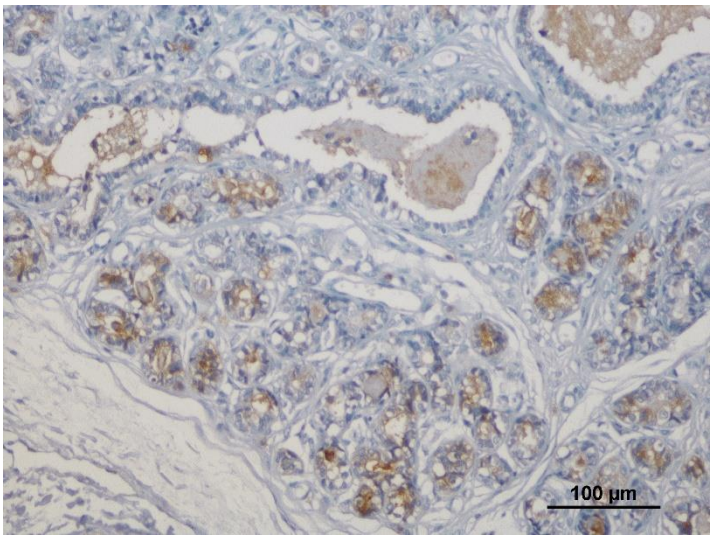

Figure 11 - Immunoreactivity for c-kit in adnexal non-tumoral mammary gland; bar = 100 μm.

= 48), whereas tumors with intermediate (28.75%, n = 23) and focal labelling patterns (11.25%, n = 9) were less frequent. For labelling intensity, there was a relatively homogeneous distribution between strong (45%, n = 36) and moderate labelling (37.5%, n = 30), whereas tumors with weak intensity (17.5%, n = 14) were less frequent. The VEGF and CD31 immunostaining followed the classic immunoreactive pattern [30]. In briefly, VEGF immunostaining was frequently observed in the cytoplasm of tumor cells and was more intense in epithelial neoplastic cells coating ducts or tubulopapillary formations with a spotted pattern. The CD31 immunoexpression and was observed in

endothelial cells as a subtle outline of microvessels. The mean number (±SD) of total CD31 was 39.12 (±20.7), minimum 8 and maximum 106.

$CD3^+$ T-lymphocytes, c-kit, VEGF and CD31 correlations

$CD3^+$ T-cells and high c-kit immunoexpression revealed a positive and significant correlation with CD31 ($r = 0.654$, $p < 0.001$; $r = 0.365$, $p = 0.003$ for CD3 and c-kit respectively) and VEGF ($r = 0.503$, $p < 0.001$; $r = 0.284$, $p = 0.023$ for CD3 and c-kit respectively). A positive correlation between $CD3^+$ T-lymphocytes and c-kit was also observed ($r = 0.263$, $p = 0.039$).

$CD3^+$ T-lymphocytes, c-kit, CD31 and VEGF expression associations

A statistically significant association between $CD3^+$ T-lymphocytes and c-kit was observed ($p = 0.039$). Tumors with high c-kit expression showed higher counts of $CD3^+$ T-cells (**Figure 12**).

The MVD of high CD3/VEGF tumors was significantly more elevated ($p < 0.001$; **Figure 13**). A similar association was observed for high c-kit/VEGF tumors ($p < 0.001$; **Figure 14**).

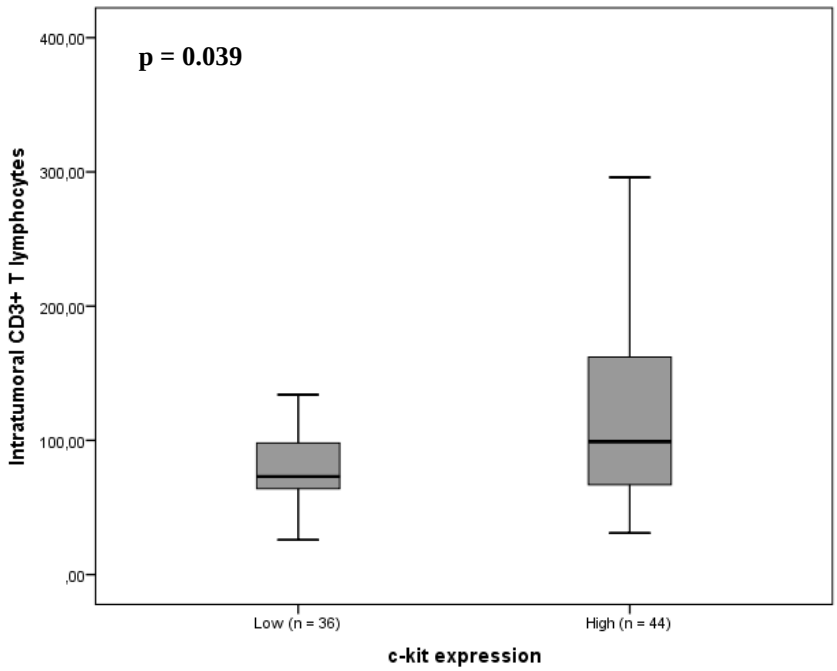

Figure 12 - Association of $CD3^+$ T-lymphocytes and c-kit in malignant canine mammary tumors.

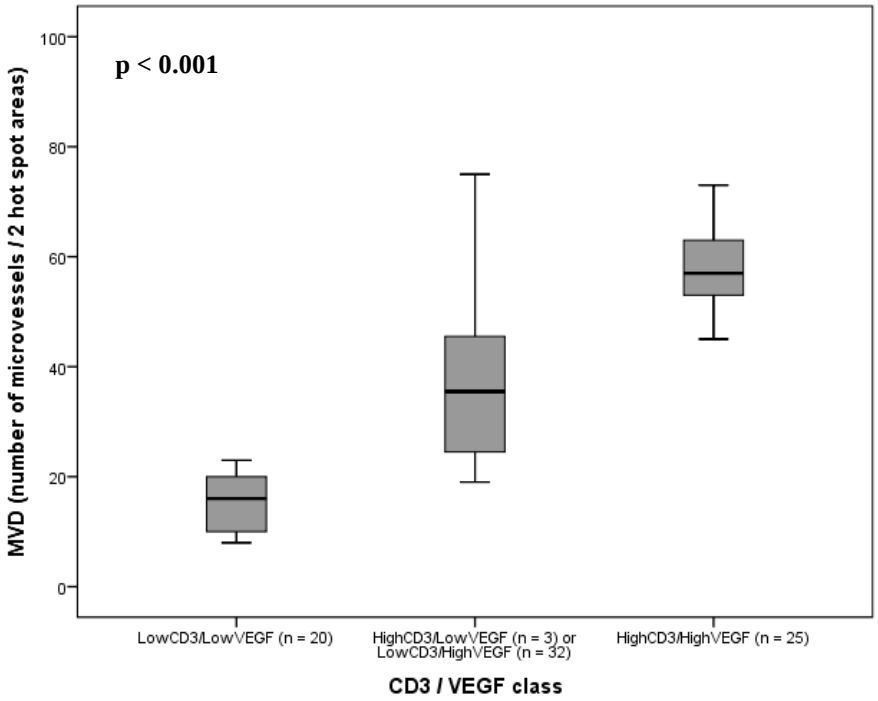

Figure 13 - Association of MVD and CD3/VEGF groups in malignant canine mammary tumors.

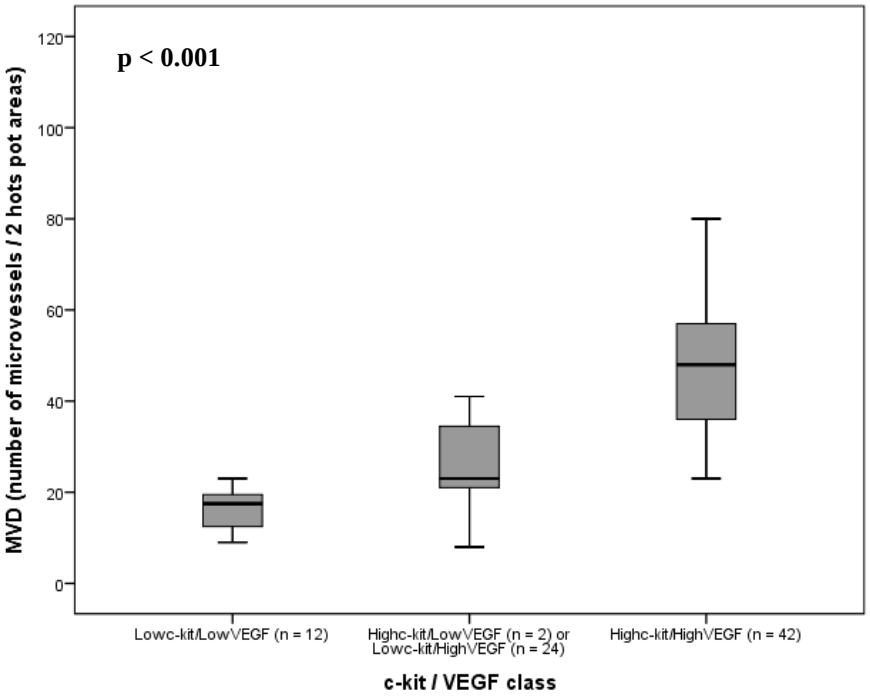

Figure 14 - Association of MVD and c-kit/VEGF groups in malignant canine mammary tumors.

Relationship of CD3/c-kit, CD3/VEGF and c-kit/VEGF with clinicopathological variables of tumor aggressiveness

In this study high CD3/c-kit, high CD3/VEGF, high c-kit/VEGF tumors were statistically associated with elevated grade of malignancy ($p < 0.001$ for CD3/c-kit, CD3/VEGF and

c-kit/VEGF), presence of neoplastic intravascular emboli ($p < 0.001$ for CD3/c-kit and CD3/VEGF; $p = 0.002$ for c-kit/VEGF) and presence of lymph node metastasis ($p < 0.001$ for CD3/c-kit, CD3/VEGF and c-kit/VEGF and). More information is provided in **Table 7**.

Comparison between CD3/c-kit, CD3/VEGF, c-kit/VEGF and OS

Tumors with high expression of CD3/c-kit ($p = 0.002$), high CD3/VEGF ($p = 0.006$), high c-kit/VEGF ($p < 0.001$) were associated with shorter OS time (**Figure 15-17**).
The group of tumors with high c-kit/VEGF retained their significance by multivariate analysis arising as independent predictor of poor prognosis (**Table 8**).

Discussion

During the course of multistep mammary tumorigenesis, tumor cells acquire functional capabilities, via distinct mechanisms, in order to survive and disseminate [34, 35]. One of the most prominent mechanisms involves the inflammatory state of premalignant and malignant mammary lesions coordinated by cells of the immune system, including T-lymphocytes [1, 2]. These immune cells, by deregulation of key signaling pathways,

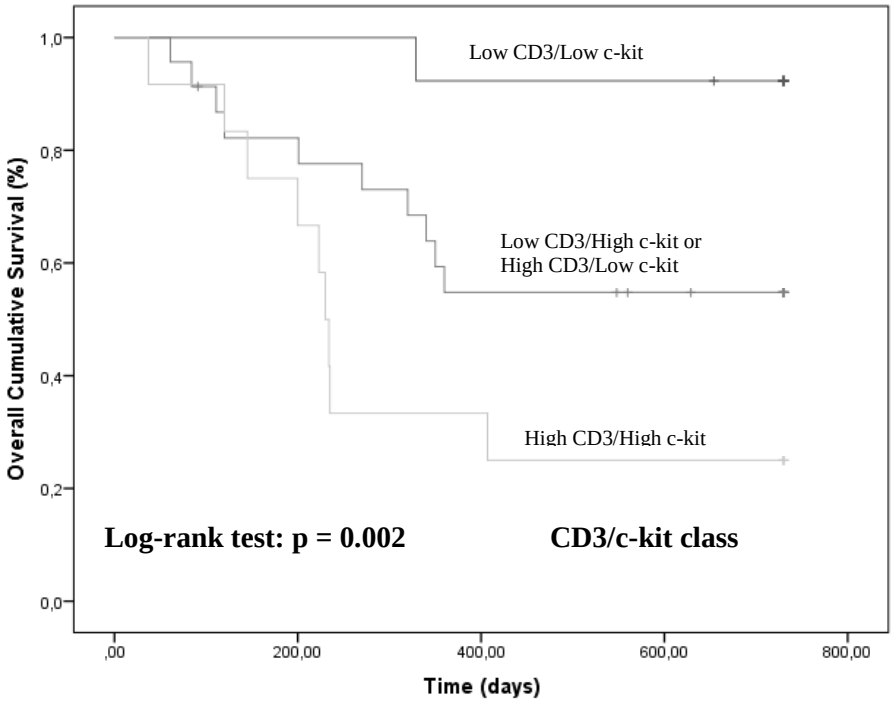

Figure 15 - Kaplan-Meier overall survival curves comparing CD3/c-kit categories in 80 dogs with malignant mammary tumors.

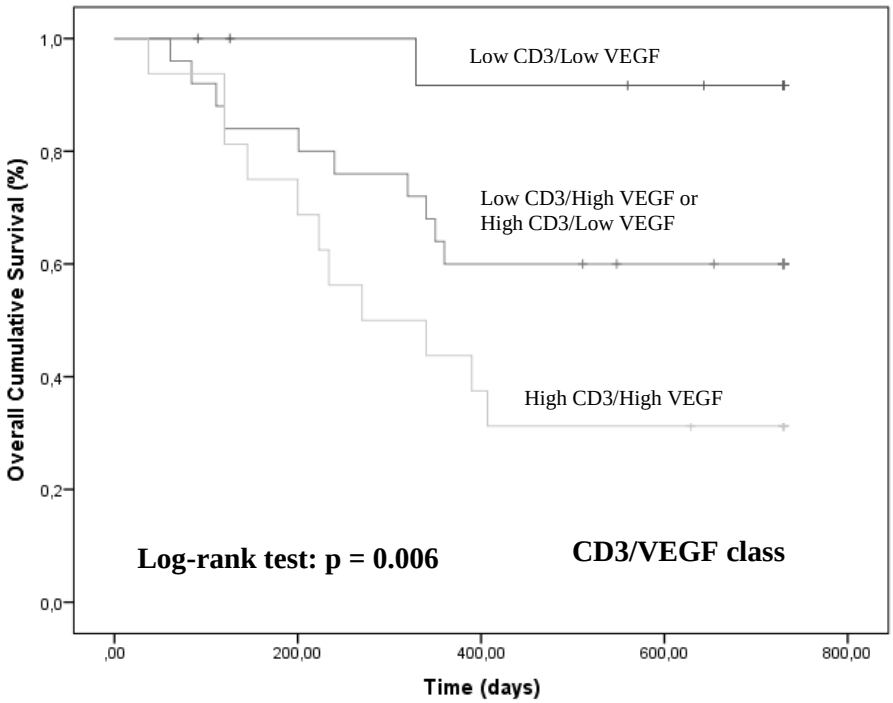

Figure 16 - Kaplan-Meier overall survival curves comparing CD3/VEGF categories in 80 dogs with malignant mammary tumors.

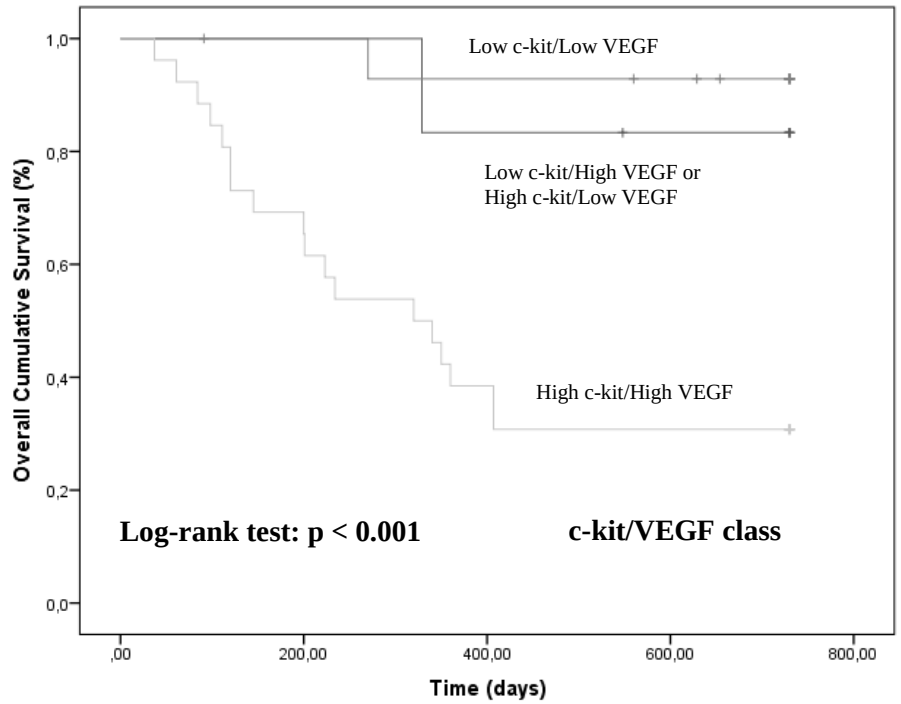

Figure 17 - Kaplan-Meier overall survival curves comparing c-kit/VEGF categories in 80 dogs with malignant mammary tumors.

contribute to human and dog mammary tumors autonomy and ability to sustain proliferative and angiogenic switch [2, 36]. The cross-pathways that happen in tumor microenvironment, are carried in large part by growth factors that bind cell-surface receptors, typically containing intracellular tyrosine kinase domains, like c-kit [16, 22,

25, 37]. Reports in both species, describe T-lymphocytes and c-kit overexpression in mammary tumours and support a role for them in tumor progression [8, 10, 22, 25].

The results of the present study demonstrate a positive correlation of CD3+ T cells and high c-kit expression with VEGF and CD31. Overall survival study revealed that high c-kit/VEGF group retained the significant relationship with OS by multivariate analysis arising as an independent predictor of poor prognosis. Angiogenesis is crucial in the growth and spread of malignant mammary tumors [38, 39]. The findings of our work are in accordance with recent literature in human breast cancer [39] and in CMT [2, 10, 11] suggesting that $CD3^+$ T-lymphocyte cytokines in mammary tumor sites may stimulate angiogenesis through the induction of pro-angiogenic factors as VEGF that contribute for new blood vessel formation from pre-existing ones and are implicated in mammary tumor aggressiveness and shorter OS times. Our results support also the possibility of common signaling pathways between c-kit and VEGF that have a key role in tumor angiogenic switch, malignancy and poor prognosis. In dogs there are only a few studies assessing the expression of c-kit in mammary tumorigenesis [25-27]. The lack of other studies in CMT and the controversy of results in human breast cancer studies [22-24] preclude more adequate comparisons of our results. However in small cell lung cancer was demonstrated that the activation of c-kit by stem cells factor (SCF) leads to a predominantly HIF-1alpha-mediated enhancement of VEGF expression and that inhibition of c-kit signaling with imatinib could result in inhibition of tumor angiogenesis [40].

Our data showed also a significant association and a positive correlation between $CD3^+$ T-lymphocytes and c-kit expression. Furthermore concurrent overexpression of these markers was statistically associated with variables of tumor aggressiveness and shorter OS. In several human tumors, including breast cancer, the SCF that triggers the c-kit signaling pathways has been described as possibly being involved in differentiation, migration, survival, and maturation of T-cells and other inflammatory cells into tumor sites, by a complex relation between mast cells, tumor cells, and T-lymphocytes in the tumor microenvironment [15, 16]. In humans, the upregulation of c-kit on dendritic cells via, induce the activation of PI3 kinase signaling that block IL-12 and promote IL-6 serum production, which in turn supports an immune twisting toward Th2 and Th17 subsets and away from Th1 responses. The Th2 and Th17 cytokines induce T-cell tolerance which is a deleterious immune condition in a variety of diseases, including cancer [15, 17, 18]. All of these immune functions attributed to c-kit disclose the complex relation between c-kit/SCF axis, tumor cells and T-cells in the tumor microenvironment that could result in

chronic inflammation with immune balance from activation to tolerance and may be implicated in mammary tumorigenesis. Therefore, the c-kit/SCF pathways are not only important for the remodeling of tumor microenvironment but also could be a very important target for tumor immunological therapy.

Conclusion

Results of this study strongly suggest that T-lymphocytes may share common signaling pathways with c-kit and VEGF in CMT progression and may contribute to increased angiogenesis, tumor aggressiveness and poor prognosis.

Table 7 - Relationship of CD3/c-kit, CD3/VEGF and c-kit/VEGF groups with clinicopathological variables of tumor aggressiveness

Molecular markers		Variables of Tumor Aggressiveness								
		HGM			Neoplastic intravascular emboli			Lymph node metastasis		
		Low (I/II)	High (III)	p	Absent	Present	p	Absent	Present	p
CD3/c-kit	Low CD3 / Low c-kit	18	4	**<0.001**	20	2	**<0.001**	18	4	**<0.001**
	Low CD3 / High c-kit or High CD3 / Low c-kit	19	16		27	8		24	11	
	High CD3 / High c-kit	3	20		8	15		7	16	
CD3/VEGF	Low CD3 / Low VEGF	17	3	**<0.001**	18	2	**<0.001**	16	4	**<0.001**
	Low CD3 / High VEGF or High CD3 / Low VEGF	21	14		28	7		26	9	
	High CD3 / High VEGF	2	23		9	16		7	18	
c-kit/VEGF	Low c-kit / Low VEGF	10	2	**<0.001**	12	0	**0.002**	11	1	**<0.001**
	Low c-kit / High VEGF or High c-kit / Low VEGF	20	6		22	4		21	5	
	High c-kit / High VEGF	10	32		21	21		17	25	

n, number of samples; p, statistical significance; NS, not significant

Table 8 - Association between considered molecular markers groups and overall survival.

Molecular markers		n	Overall Survival Univariate (Mean values)	p	Overall Survival Multivariate* (Hazard ratio)	p
CD3/c-kit	Low CD3 / Low c-kit	22	699.154	**0.002**	-	NS
	Low CD3 / High c-kit or High CD3 / Low c-kit	35	500.809			
	High CD3 / High c-kit	23	335.083			
CD3/VEGF	Low CD3 / Low VEGF	20	696.583	**0.006**	-	NS
	Low CD3 / High VEGF or High CD3 / Low VEGF	35	524.480			
	High CD3 / High VEGF	25	383.500			
c-kit/VEGF	Low c-kit / Low VEGF	12	663.167	**<0.001**	**6.102** (CI: 0.807 - 46.152)	**0.010**
	Low c-kit / High VEGF or High c-kit / Low VEGF	26	697.143			
	High c-kit / High VEGF	42	371.462			

n, number of samples; p, statistical significance; NS, not significant; CI, confidence interval; *Multivariate Cox Proportional Hazard Analysis (Cox Regression) of overall survival (OS) in dogs with malignant mammary tumors in a prospective study with 2 years of follow-up.

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Chapter IV – The Potential Crosstalk between Tumor Cells and Immune Cells on COX-2 Regulation: an *in vitro* Co-Culturing Experiment

Original Research Articles:

Bidirectional Regulation of COX-2 expression between Cancer Cells and Macrophages. Maria Isabel Carvalho. Rodolfo Bianchini, Judit Fazekas, Ina Herrmann, Irene Flickinger, Johann G. Thalhammer, Isabel Pires, Erika Jensen-Jarolim, Felisbina Luísa Queiroga. Submitted manuscript

IV.1 Bidirectional Regulation of COX-2 expression between Cancer Cells and Macrophages

Abstract

Background: COX-2 expression in mammary cancer cells correlates with cancer progression and tumor associated inflammation including M2 macrophages which support the immunosuppressive environment in cancer.

Aims: The purpose of this study was to investigate a potential crosstalk of tumor cells and immune cells and its effects on COX-2 regulation in canine mammary cancer (CMC).

Methods: Canine mammary cancer Sh1b cell, compared to human mammary BT474 or human colon HT29 cancer cells were co-cultured with canine peripheral blood mononuclear cells (PBMCs) or with macrophage-like differentiated THP1 monocytes (dTHP1) during 48h using transwell inserts. The expression of intracellular COX-2 by PBMCs or dTHP1, and on the other hand in the cancer cells, was evaluated by flow cytometry. Further, medium was collected from cultured Sh1b and HT29 cancer cells after 24h culture for conditioning of dTHP1 monocytes during 48h.

Results: Co-culturing of Sh1b and canine PBMCs induced a trend of COX-2 overexpression in mammary cancer cells. In turn, COX-2 expression by PBMCs, among them predominantly $CD68^+$ macrophages, was significantly attenuated by co-culture with Sh1b ($p = 0.0001$). In accordance, co-culture with dTHP1 prompted an intracellular production of COX-2 both in Sh1b CMC cells and HT29 human colon cancer cells and a decreased production of COX-2 in BT474 human mammary cancer cells. The intracellular COX-2 expression from dTHP1 decreased when they were treated with conditioned medium from cultured Sh1b and HT29 cancer cells.

Conclusion: Our results highlight the bidirectional COX-2 regulation between cancer and monocytes/macrophages in CMC, which together shape the tolerogenic tumor microenvironment.

Introduction

Tumor progression is critically dependent on the microenvironment in the surrounding stroma. The microenvironment contains a cocktail of inflammatory and regenerative cells and immune cells, along with all cytokines and mediators released by them that may support or suppress tumor growth [1].

T-lymphocytes and macrophages are major constituents of the inflammatory infiltrate observed in several human and canine tumors. In both species, these immune cells in mammary tumor sites produce a variety of inflammatory mediators that influence tumor progression, invasion and metastasis [2-6]. Interestingly, animal and human studies report that especially COX-2 overexpression in mammary tumors strongly support the hallmarks of cancer and disease progression [7-12].

In parallel, in human and canine mammary tumors, also the presence of high numbers of intratumoral T-lymphocytes as well as of macrophages with a typical M2 phenotype are mostly associated with poor overall survival (OS) [4, 13, 14]. Immunosuppression associated with T-lymphocyte infiltration has been explained by the compartment of $CD4^+CD25^+FoxP3^+$ regulatory T-cells that inhibit any cytotoxic activities [15-18]. In contrast, the immunosuppressive and hence pro-tumor action of macrophages is much less defined. The interplay of macrophages and tumor cells was hence in the focus of our study, especially their possible mutual effects on COX-2 expression, for the following reasons:

In human breast cancer COX-2-derived prostaglandin E2 (PGE2) has the ability to influence local immune responses in the tumor stroma contributing to tumor evasion of immune surveillance [11, 19]. A substantial body of work describes an immunosuppressive role for COX-2. The COX-2/PGE2 signaling pathways may contribute to the modulation of macrophages inducing the promotion of M2-macrophages cytokines which nourish Th2 immune responses [20, 21]. The immune cell subtypes modulated by COX-2 share common cytokine mediators and more importantly, each cell subtype can also influence the COX-2 production, providing an autocrine mechanism for prolonging and enhancing their own immunosuppressive phenotype [22].

Collectively, these findings show that COX-2 in cancer and immune cells share common signaling pathways. This is relevant for mammary carcinogenesis associated with changes in immune cell profiles and functionality, possibly allowing neoplastic cells to evade attack by the immune system [22].

In contrast, in canine mammary tumors only few studies have yet concentrated on the COX-2 crosstalk between cancer and immune cells [23, 24]. We therefore aimed here to contribute to the understanding of a potential bidirectional crosstalk between macrophages and cancer cells and the role of COX-2 expression.

Material and Methods

Cell culture

Canine mammary carcinoma cell line Sh1b was a kind gift of Dr. Gerard Rutteman (Department of Clinic Science and Companion Animals, University of Utrecht, The Netherlands) to the University of Veterinary Medicine Vienna. Human colon cancer cell line HT29, human breast carcinoma cell line BT474 and human monocytic cell line THP-1 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Sh1b was maintained in DMEM F12 (Gibco, Invitrogen) supplemented with heat inactivated 10% fetal calf serum (FCS), 2mM L-glutamine and 10µg/mL gentamicin sulfate. BT474 was maintained in DMEM (Gibco, Invitrogen) with 10% FCS, 2mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml). HT29 and THP1 were cultured in RPMI-1640 (Gibco, Invitrogen) supplemented with 10% FCS, 2mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were incubated at 37ºC in a humidified atmosphere containing 95% air and 5% CO_2.

Collection of tumor cell line cultured supernatants

Sh1b, HT29 and BT474 cells were grown in flasks at 80-90 % confluence and harvested with trypsin. After that, 8 x 10^5 cells/mL were seeded into 6 well culture plate containing 2 mL of DMEM F12 (for Sh1b), 2mL of DMEM (for BT474) or 2 mL of RPMI-1640 (for HT29) per well. Cells were incubated at 37ºC in a humidified atmosphere containing 95% air and 5% CO_2. After 24h incubation the medium of all cell lines was changed for fresh RPMI-1640 and the cultured supernatants were collected 24h later and stored at -20ºC until needed for the corresponding experimental conditions.

THP1 differentiation in response to PMA and treatment with cancer cell cultured supernatants

THP1 cells (0,2 x 10^6 cells/ml) were seeded into a 24 well plate containing 500 µL of RPMI-1640 culture medium in the presence of 200 nM of Phorbol myristate acetate

(PMA) for 72h. After incubation, nonattached cells were removed by aspiration. The adherent THP1 cells were washed three times with Hank's Balanced Salt Solution (HBSS) to completely remove PMA and treated with 50% v/v of Sh1b or HT29 cultured supernatants during 48h.

Isolation of dog peripheral blood mononuclear cells (PBMCs)

Blood samples from male and female spayed dogs (n = 11) received for cancer treatment in the Oncology Clinic of the University of Veterinary Medicine Vienna were used in this study. All dogs received doxorubicin-derived chemotherapy and at the blood collection the treatment duration was less than 3 months. Two milliliters of blood was drawn from the cephalic vein of each dog by venipuncture into EDTA tubes. Dog PBMCs isolation procedure was performed with the standard Ficoll-Paque solution (Sigma Chemical, St. Louis, MO) density gradient centrifugation. After collect all the content of the milky PBMCs layer, cells were washed in HBSS, re-suspended in DMEM, counted and incubated in RPMI-1640 culture medium.

Co-culture experimental conditions

Sh1b, HT29 and BT474 cell lines (5×10^5 cells per well in the co-culture day) were plated into a 24 well plate in respective culture medium. On day 2 the culture medium of Sh1b, HT29 and BT474 was changed for fresh RPMI-1640 supplemented with 10% FCS, 2mM glutamine and penicillin/streptomycin antibiotic. Canine PBMCs (1×10^6 cells/ml) or dTHP1 ($0{,}2 \times 10^6$ cells/ml) were plated directly on the 24 well plate transwell inserts (0.4 µm pore size, Corning, Life sciences, MA) in RPMI-1640 culture medium and co-incubated with canine mammary cancer cell line Sh1b during 48h. The PMA dTHP1 macrophages were also co-incubated with HT29 and BT474 human cell lines.

Assessment of CD68, CD3 and intracellular COX-2 by Flow Cytometry

The CD68, CD3 and intracellular COX-2 expression in canine PBMCs; the intracellular COX-2 expression in Sh1b, HT29 and BT474; the CD68 and intracellular COX-2 expression in dTHP1 were evaluated on a FACSCantoII flow cytometer (BD Biosciences, Franklin Lakes, NJ).

Briefly, all cells were detached, washed three times with HBSS, and re-suspended in FACS buffer. After that, the primary antibodies master mix CD68 (clone eBioY1/82A;

PE-Cy7; eBioscience, San Diego, CA; 2µl per tube) and CD3 (clone CA17.2A12; purified; Biorad, Hercules, CA; 1µl per tube) were added and cells were incubated 30 min at 4°C in the dark. Subsequently cells were washed with FACS buffer and incubated 30 min at 4°C (in the dark) with rat anti-mouse IgG1 secondary antibody for CD3 (clone M1-14012; APC; eBioscience, San Diego, CA; 1µl per tube). After washing, cells were fixed with IC fixation buffer (eBioscience, San Diego, CA) and permeabilized with permeabilization buffer (eBioscience, San Diego, CA) according to the manufacturer's instructions and was added the COX-2 primary antibody (SP21, Thermo Scientific, Lab Vision; 1:50 dilution) for 30 min at 4°C. Then, cells were washed in permeabilization buffer and incubated 30 min at 4°C (in the dark) with secondary antibody for COX-2 (Goat anti-rabbit IgG; AF488; Life Technologies; Waltham, MA, USA; 2µg/mL). Finally, cells were washed with permeabilization buffer three times, re-suspended in FACS buffer and analyzed by flow cytometry. An appropriate isotype control was used to adjust for background fluorescence, and results are reported as the % of expression or as the geometric mean fluorescence intensity (MFI). Flow cytometry analysis was performed using the data analysis software FlowJo version X (FlowJo LLC, Ashland, OR).

Statistical analysis

Statistical software SPSS (Statistical Package for the Social Sciences, Chicago, IL, USA) version 19.0 was used for statistical analysis. Comparisons among groups were performed with ANOVA or Student's *t*-test. Only values of $p < 0.05$ were considered significant.

Results

Co-culturing canine cancer Sh1b and canine PBMCs from tumor patients: effects on COX-2

To determine whether COX-2 expression in canine mammary carcinoma cells was co-regulated by immune cells, we performed an in vitro co-culturing experiment. The co-cultured SH1b mammary cancer cells with canine PBMCs showed a trend of overexpression of COX-2 in comparison with SH1b alone (**Figure 1 A**). The expression of COX-2 by the PBMCs was significantly attenuated by the co-culture with the cancer cell line ($p = 0.0002$; **Figure 1 B**). Interestingly, $CD68^+$ cells, putatively macrophages, are characterized by the highest expression of COX-2 among PBMCs. The co-culture

between Sh1b and dog PBMCs induced a decreased expression of intracellular COX-2 by the $CD68^+$ fraction in the canine PBMCs (p = 0.0215; **Figure 1 C**), but did not induce considerable alterations in intracellular COX-2 expression by $CD3^+$ PBMCs (**Figure 1 D**).

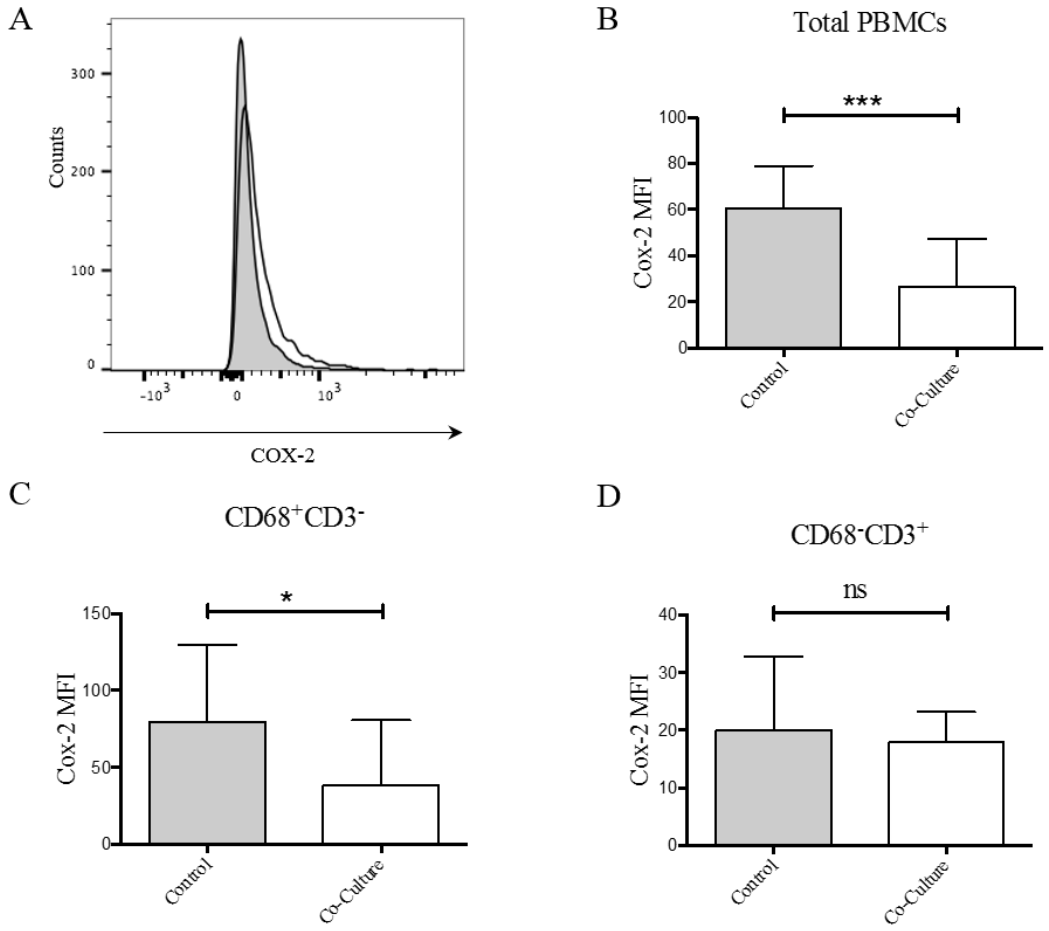

Figure 1 - Effects of co-culture of PBMCs and canine cancer cells on intracellular COX-2 expression. (A) Graphs show the overlay of flow cytometric expression of intracellular COX-2 by Sh1b mammary cancer cells (gray filled histogram) or Sh1b cancer cells in co-culture with canine PBMCs (black solid line). Co-cultured Sh1b mammary cancer cells with canine PBMCs showed a trend of overexpression of COX-2 in comparison with SH1b alone. The co-culture between Sh1b mammary cancer cells and canine PBMCs results in **(B)** a decreased expression of intracellular COX-2 by the PBMCs (*** p = 0.0002), **(C)** induced a decreased expression of intracellular COX-2 specifically in the fraction of $CD68^+CD3^-$ cells within the PBMCs (* p = 0.0215), but **(D)** did not induce alterations in COX-2 expression in the $CD68^-CD3^+$ cells. Graphs show the mean of 5 independent experiments +/- SD. P-values were calculated by two tail paired Student t-test: ns= not significant, *p<0.05, **p<0.01, ***p<0.001).

Co-culturing cancer cell lines and human macrophages dTHP1: effects on COX-2

The results obtained with PBMCs were validated in the $CD68^+$ monocytic cell line THP1. Co-culturing with dTHP1 indeed prompted an up-regulation of COX-2 in the canine mammary cancer cells Sh1b (**Figure 2 A**).

Against our expectations, co-culture of dTHP1 prompted a decreased intracellular production of COX-2 in the human mammary cancer cells BT474 (**Figure 2 B**).

In accordance with the results obtained with Sh1b, co-culture between human colon cancer HT29 and dTHP1 induced an increase of COX-2 expression in the human colon cancer cells (**Figure 2 C**).

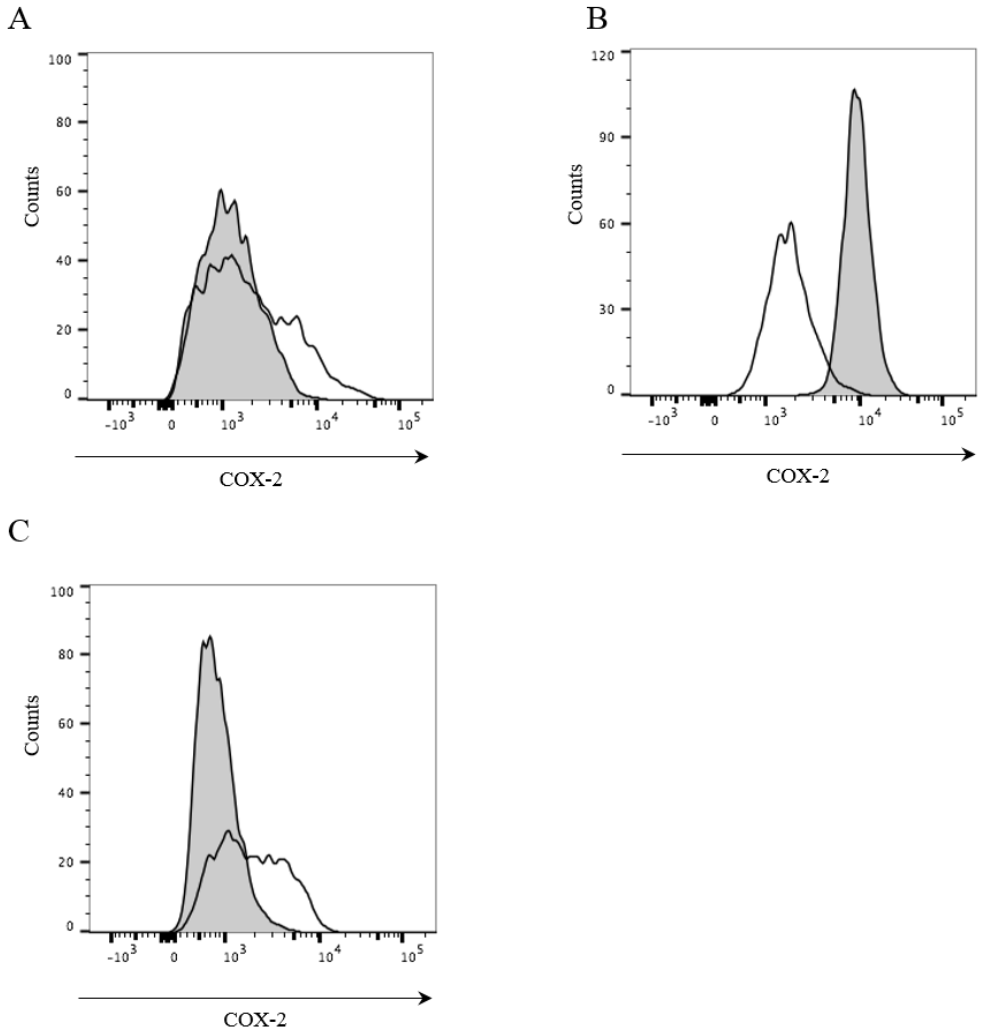

Figure 2 - The effects of co-culture between human macrophage cell line and canine or human cancer cells lines on COX-2 expression by the cancer cells. Co-cultured dTHP1 with canine mammary cancer cell Sh1b **(A)**, result in overexpression of intracellular COX-2; with human mammary cancer cell BT474 **(B)**, results in a decreased intracellular production of COX-2; and with human colon cancer cell HT29 **(C)**, result in overexpression of intracellular COX-2. Graphs show the overlay of flow cytometric expression of intracellular COX-2 by cancer cells (gray filled histogram) or cancer cells in co-culture (black solid line).

Effects of cancer cell supernatants on intracellular COX-2 expression by dTHP1

In the co-culture model used above, immune cells are not allowed to make direct contact with the tumor cells. Thus to demonstrate the presence of soluble factors that might

regulate intracellular COX-2 expression in the monocytic dTHP1, cells were treated with conditioned medium recovered from Sh1b and HT29 cells cultured for 48h. The dTHP1 treated with both Sh1b and HT29 culture supernatants, showed a decrease of intracellular COX-2 expression (**Figure 3**).

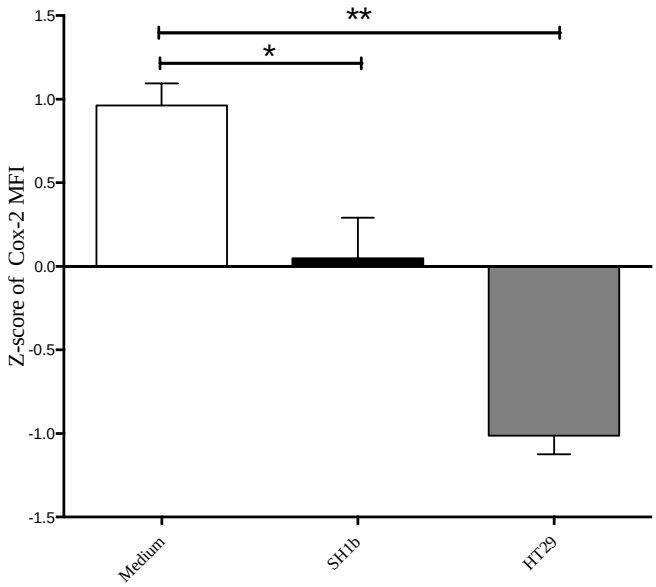

Figure 3 - Effects of soluble factors derived from cultured cancer cells on intracellular COX-2 expression by macrophages. dTHP1 macrophages were cultured in medium alone (white column) or treated with 50% v/v conditioned medium from cultured canine mammary cancer cells Sh1b (black; * p = 0.0257) or human colon cancer cells HT29 (gray; ** p = 0.0028) for 48h. Graph shows the z-score of intracellular COX-2 MFI of 2 independent experiments +/- SD. P-values were calculated by one-way ANOVA with Bonferroni's multiple comparisons post test: *p<0.05, **p<0.01.

Discussion

In human mammary tumors COX-2 has crucial effects on the regulation and activity of T-cells and macrophages [19, 20, 25-27] contributing to tumor evasion of immune surveillance and supporting tumor development and metastasis [11, 19]. Additionally in murine breast cancer models selective COX-2 inhibitors could restrain breast cancer through augmentation of Th1 anti-tumor immune responses [26, 28]. In canine mammary cancer, however, the association between COX-2 and immune cells has so far only been described by immunohistochemistry, hence in a descriptive manner [23, 24]. In order to determine the potential mutual regulation of COX-2 between immune cells and mammary cancer cells, in this study this aspect of the tumor microenvironment was mimicked *in vitro* through a transwell system. When in a first step the canine mammary carcinoma cell line Sh1b was co-cultured with canine PBMCs from tumor patients a trend of

overexpression of COX-2 was observed in comparison with Sh1b alone. In turn the expression of COX-2 by PBMCs was significantly downregulated. Almost all COX-2 expression could be allocated to $CD68^+$ cells within the PBMCs, likely representing macrophages. Importantly, the co-culture did not induce considerable alterations in intracellular COX-2 expression by $CD3^+$ PBMCs.

Clinical and experimental human studies indicate that not only tumor associated T-lymphocytes but also macrophage polarization is modulated by the tumor microenvironment in mammary cancer. In tumor microenvironment macrophages are "educated" adopting a role that facilitates angiogenesis, matrix breakdown and tumor-cell motility in a COX-2-dependent manner [5, 6, 20, 26, 29].

In accordance with the results of our work, a study using co-culture between human mammary cancer cell line HCC1954 and mouse peritoneal macrophages showed an increased COX-2 expression in the cancer cells and an elevated PGE2 levels in conditioned media. Similar results were observed when human monocyte-derived macrophages were co-cultured with HCC1954 cells [27]. More recently another study using tumor associated macrophages (TAMs) isolated from tumor tissues demonstrated that $COX\text{-}2^+$ TAMs promoted human mammary cancer cells proliferation and survival [22]. Furthermore, in the same study COX-2 in TAMs induced the expression of COX-2 in breast cancer cells, which in turn promoted polarization of macrophages towards M2, hence, a positive-feedback loop exists between macrophages and breast cancer cells [22]. Although in dogs recent studies showed the relevance of a relationship between canine mammary cancer cells and macrophages that promote the macrophage polarization and the cancer cells migration and invasion [6, 30], to our knowledge there are no studies on their potential interdependence in terms of COX-2 regulation.

In order to validate the results obtained with the $CD68^+$ cell fraction within canine PBMCs, we next used the human $CD68^+$ cell line THP1. Their biologic behavior is similar to monocyte-derived macrophages regarding adherence and phagocytic capacity, surface marker and cytokine expression [31] and they are widely used as monocyte/macrophage model [22, 31, 32]. Previously, the co-culture with THP1 and HCC1937 human mammary cancer cell line led to increased COX-2 expression in cancer cells [22]. In accordance, our results demonstrated that the co-culture with dTHP1 prompted an intracellular production of COX-2 both in Sh1b canine mammary cancer cells and HT29 human colon cancer cells, but unexpectedly a decreased production of COX-2 in human mammary cancer cells of the BT474 type. The effects were independent

on cellular contact, but were transmitted by soluble mediators, from three different canine and human cancer cell lines to the monocytic cell line THP1.

We would like to emphasize that THP1 is a model cell line only. Therefore, we find it important that they mirrored the results from the $CD68^+$ cell fraction within PBMCs from canine cancer patients. Also, other researchers demonstrated that COX-2 in TAMs induced the expression of COX-2 in cancer cells [20, 21]. Importantly, this in turn, promoted macrophage polarization to the M2 tumor supportive phenotype [27].

Taken together, the COX-2-mediated immunomodulation in human breast cancer is a phenomenon shared by cancer cells and macrophages which supports our data. This emphasizes the importance of exploring the tumor microenvironment as a whole [1, 22]. The findings of the present study suggest for the first time that similarly to human breast cancer, also in canine mammary cancer COX-2 regulation in tumor cells and macrophages are interconnected and dependent on yet unidentified soluble molecules.

Conclusion

Present data highlight the bidirectional COX-2 regulation in canine mammary microenvironment, implicating the contribution of cancer cells and macrophages. Immune cells-mediated induction of COX-2 in canine mammary cancer cells may provide a mechanism whereby tumor associated inflammatory cells contribute to tumor progression. The COX-2 regulation between cancer cells and macrophages in canine mammary cancer contribute to shape the tolerogenic tumor microenvironment and understanding this phenomenon might enlighten new potential therapeutic targets in canine mammary tumors.

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Chapter V - General Discussion and Concluding Remarks

Neoplastic diseases are the most frequent clinical occurrence in the mammary gland of the female dogs. Over the decades, the interest in studying the carcinogenesis and prognosis of canine mammary tumors (CMT) has been increasing [1-6], due to the high percentage of malignant cases in dogs. Additionally, the importance attributed by several authors to CMT as a biological model for the study of human breast cancer has contributed to the growing interest in this subject [7-12]. The prognosis is usually established in agreement with tumor aggressiveness parameters (presence of skin ulceration, histological grade of malignancy, presence of lymph node metastasis, presence of neoplastic intravascular emboli); however, in order to provide additional information in the diagnosis and prognosis, recent studies have been conducted to discovery new molecular markers [6, 13].

The tumor microenvironment is highly complex and include several cells, as well as the biological products released by them. The tumor infiltrating immune cells are widely accepted to be generic constituents of tumor microenvironment [14, 15]. In humans it is well established that the development of mammary cancer is associated with alterations in numbers and functions of immune cells at the tumor sites [16-18]. In fact, mammary cancer cells can take advantage of signaling molecules released by tumor-infiltrating immune cells, which exhibit exclusive phenotypic and functional characteristics [19].

Cancer related inflammation has an important role in mammary carcinogenesis. It contributes to the acquisition of hallmark capabilities that allow cancer cells to survive, proliferate, and disseminate which is related with aggressiveness and poor clinical outcome [20, 21]. Recent studies in human breast cancer have already identified a growing list of signaling molecules released by inflammatory cells that serve as effectors of their tumor-promoting actions [22-25].

In dogs there are some studies about the influence of the immune system in mammary carcinogenesis [26-31], however only few studies have focused on the role of immune cells in clinical outcome of dog mammary tumors patients [26, 28, 29, 32]. In this context, the present work was conducted to understanding the roles of the intertwined signaling pathways shared by T-lymphocytic/macrophage infiltrates and important tissue biomarkers in CMT progression, aggressiveness and prognosis. This molecular interplays might enlighten new prognostic factors and molecular therapeutic targets that could be valuable in developing clinically effective immunotherapeutic approaches.

Similarly to human breast cancer studies, it was already described in CMT a relationship of tumor-infiltrating T-lymphocytes (TILs) and mammary tumors malignancy [28], an

association of ratio $CD4^+/CD8^+$ T-cells with low survival rates [26], a promotion of tumor progression by Th2 cells actions [33] and an association of great amounts of Treg cells with clinicopathological factors of tumor aggressiveness [29, 30]. Immunosuppression associated with TILs has been explained by the compartment of $CD4^+CD25^+FoxP3^+$ regulatory T-cells that inhibits anti-tumor cytotoxic activities [34-37]. Based on these evidences, first, we assess the characterization of intratumoral Treg cells immunoexpression, as well as some cytokines related by them (TGFβ and IL-35) and attempt to clarify the role of these molecular markers in CMT aggressiveness and prognosis. In the present study, we demonstrated that higher values of FoxP3 were present in tumors with more aggressive phenotypes (high HGM, presence of neoplastic intravascular emboli and presence of lymph node metastasis) and our results are in agreement with other studies in human [38] and dog mammary tumors [30, 32]. Additionally, present work demonstrated that the higher numbers of $FoxP3^+$ Treg cells within the intratumoral compartment were associated with shorter OS of animals, both in univariate and multivariate analysis, which is an innovative finding not described previously. These findings prove the value of $FoxP3^+$ Treg cells as an independent prognostic factor in CMT and support the hypothesis that Treg cells allows the tumor cells to evade the anti-tumor immune responses, facilitating tumor progression and development. In humans there are studies that revealed a negative role of Treg cells in the prognosis of breast cancer patients [35, 39, 40] and the independent prognostic value of these immune cells was also demonstrated [41]. Recently a study demonstrated in CMT a significant association of Tregs and poor disease-free survival time by univariate survival analysis [32]. Treg cells have the capacity to secrete inhibitory cytokines, such as IL-10 and TGFβ inducing tumors tolerance [36, 42, 43]. The TGFβ potent immunosuppressive signaling represses T-cells inflammatory and cytotoxic differentiation programs [44, 45]. In addition to impairing T-cells effector functions, TGFβ plays a pivotal role in the generation of Treg cells from a population of peripheral $CD4^+CD25^-$ T-cells through the induction of key transcription factor FoxP3 [46, 47]. In human breast cancer the TGFβ and FoxP3 overexpression has a crucial impact in several tumor hallmark steps, including angiogenesis which induces tumor progression, dissemination and metastasis [45, 46, 48]. Present work suggests a link between TGFβ and more aggressive tumor phenotypes, reflecting its involvement in CMT malignant transformation accordingly with recent

literature in human breast cancer [49-53]. Our data demonstrated also a positive correlation between intratumoral FoxP3, TGFβ levels, VEGF and CD31. Moreover, tumors with abundant TGFβ and with concurrent high expression of TGFβ/FoxP3, FoxP3/VEGF, TGFβ/VEGF were associated with shorter OS time and TGFβ/FoxP3 tumor class retained the association with worse survival in multivariate analysis, arising as an independent predictor of poor prognosis. Our results are in accordance with recent data in human mammary tumors that proved the role of TGFβ and FoxP3 in VEGF signaling and in tumor angiogenic switch with an increased intratumoral microvessel density, contributing to mammary carcinogenesis and poor prognosis [48].

The increasingly high concentrations of TGFβ secreted by tumor cells induces and stabilizes the transcription factor FoxP3 expression in peripheral $CD4^+CD25^-$ T-cells and their precursors, rendering them inactive [46, 54]. Moreover, Treg cells increases the TGFβ effects creating a positive auto-regulatory loop of TGFβ signaling in $CD4^+CD25^-$ T-cells that possibly stabilizes their regulatory phenotype [47].

In humans, studies demonstrated that TGFβ and FoxP3 common signaling pathways have a crucial impact in several phases of mammary carcinogenesis, including tumor angiogenic switch [45, 46, 48]. TGFβ and FoxP3 are reported to be sufficient to upregulate the expression of VEGF, which is one of the most selective and potent angiogenic factors known, attracting adjacent endothelial cells and promoting the formation of tumor neovascularization [48, 55].

As already described above, Treg cells suppress other immune effector cells by numerous mechanisms, such as the secretion of inhibitory cytokines [56-58]. IL-35 is a Treg cell-secreted cytokine that inhibits T-cells proliferation and function [59]. In human breast cancer patients it was observed a significant increase of IL-35 expression [60] and findings suggest that IL-35 seems to be an important indicator of breast cancer progression [61]. In CMT, to the best of our knowledge, this is the first study that investigate the role of IL-35 in canine mammary tumorigenesis, cancer development and clinical outcome. According with published findings in human breast cancer [60, 61], our results showed that IL-35 overexpression in CMT was significantly associated with progression of tumor stage (high HGM, presence of neoplastic intravascular emboli and presence of lymph node metastasis) and unfavorable prognosis by univariate and multivariate analysis.

In the tumor microenvironment IL-35 induces the conversion of proliferative $FoxP3^-$ conventional T-cells into a hyporesponsive, strongly suppressive IL-35-producing

$CD4^+FoxP3^-$ induced regulatory T-cell population (iTr35 cells) which has been found to potently inhibit anti-tumor T-cell responses [56, 59, 60]. IL-35 is required for the maximum regulatory activity of human and mouse Treg cells under both *in vitro* and *in vivo* conditions. iTr35 cells population which is strongly suppressive, does not express FoxP3, does not require other key suppressive cytokines (IL-10 or transforming growth factor β, TGFβ) and/or additional molecules for conversion and optimal generation [56, 60]. Our results suggest that, in CMT, similar signaling pathways could be present and IL-35 may be a new biomarker for the prognosis of CMT patients.

For a more comprehensive approach about the role of immune cells in dog mammary carcinogenesis, we perform a wide study focused on the intertwined signaling pathways shared by T-lymphocytic/macrophage infiltrates and important tissue biomarkers in mammary tumor microenvironment. We focus in the most important cell biomarkers in mammary carcinogenesis, the COX-2, EGFR and c-kit, which are often overexpressed or mutated in this type of tumor [12, 62-65].

Recent findings revealed that COX-2-produced prostaglandins are potent lipid molecules that act as immunomodulators in key aspects of mammary tumor immunity [66-69]. Evidences from clinical and experimental studies indicate that COX-2 play critical roles on T-cells and macrophages responses in order to promote breast cancer progression and metastasis [68-70]. In human breast cancer PGE2 induces suppression of antigen-presenting dendritic cells, leading to a reduced activation of anti-tumor cytotoxic $CD8^+$ T-cells [71, 72]. Furthermore, it has been reported to enhance pro-tumorigenic Th2 responses. [73] The relationship between the deregulation of PGE2 intracellular metabolism and macrophages differentiation into the M2 subtype, related to mammary carcinogenesis phenomenon, is also verified [68, 74].

Present data demonstrated a significant association of high COX-2 immunoexpression with $CD3^+$ T-lymphocytes and MAC387 macrophages. Tumors with concurrent high COX-2/CD3 and high COX-2/MAC expression were associated with variables of tumor aggressiveness (high HGM, presence of neoplastic intravascular emboli and presence of lymph node metastases) and shorter OS. These findings suggest that, similarly to human breast cancer, also in CMT, T-lymphocytes, macrophages and COX-2 share functions in mammary carcinogenesis.

In human breast cancer, recent findings suggests that COX-2 has been reported to up-regulate the EGFR by a positive feedback loop [75]. A positive and significant correlation between COX-2 and EGFR in CMT was also demonstrated by our team. Additionally,

tumors with high COX-2 and EGFR immunoexpression were statistically associated with tumor aggressiveness [76].

COX-2/EGFR upregulated pathways have been described as a major determinant for breast cancer progression and metastasis largely due to the ability to regulate and suppress the cytotoxic responses of the immune system [69, 77-79].

Interestingly, similarly to human breast cancer findings, current results demonstrated that the concurrent COX-2^+/$EGFR^+$ expression was significantly associated with higher numbers of intratumoral $CD3^+$ T-cells. According to the present results, tumoral $CD3^+$ T-lymphocytes may be influenced by inappropriate expression of COX-2/EGFR. COX-2 overexpression and the resulting increase in PGE2 levels could induce upregulation of EGFR, possibly representing a strategy adopted by tumors that contributes to the evasion of tumor-specific immune response.

In human breast cancer, c-kit signaling pathways have also been described as possibly being involved in the complex relationship between immune cells and tumor cells in the tumor microenvironment [80, 81]. The c-kit dependent signaling pathways support an immune twisting toward Th2 and Th17 subsets and away from Th1 responses. Moreover, the cytokines produced induces T-cells tolerance and contributes for cancer development and angiogenesis [64, 80, 82-88].

Present results demonstrated a significant association and a positive correlation between $CD3^+$ T-lymphocytes and c-kit expression. Tumors with high c-kit expression showed higher counts of $CD3^+$ T-cells and were statistically associated with variables of tumor aggressiveness (high grade of malignancy, presence of neoplastic intravascular emboli and presence of lymph node metastasis), angiogenesis (VEGF and CD31) and shorter OS. To our knowledge, there are no studies in human breast cancer and in CMT that focus on relationship between T-lymphocytes, c-kit expression and mammary tumors angiogenesis and aggressiveness. The possible common signaling/regulatory pathways between c-kit and T-lymphocyte in these tumors may provide new insights into the role of c-kit in inflammation, immunosuppression and tumor progression.

The findings described above prove that COX-2, EGFR and c-kit pathways are important not only for the remodeling of mammary tumor microenvironment but also could be a very important targets for tumor immunological therapy.

Amongst the important cell biomarkers in mammary carcinogenesis, COX-2 seems to be subject of scientific community special attention [89-93]. The interest of doing studies in the scope of COX-2 expression is enhanced by the availability of selective COX-2

inhibitors which are an alternative for the treatment of mammary neoplasias in dogs and can be used alone or combined with other basic therapies such as antineoplastic chemotherapy and immunotherapy [93-96]. In this context and based on these evidences, in the present study our aim was to prove, in an *in vitro* co-culturing experiment, the potential bidirectional crosstalk between tumor cells and immune cells on COX-2 regulation. Our data showed that co-culturing of canine mammary carcinoma cell line Sh1b and canine PBMCs induced a trend of COX-2 overexpression in mammary cancer cells. In turn, COX-2 expression by PBMCs, among them predominantly $CD68^+$ macrophages, was significantly attenuated by co-culture with Sh1b. In accordance, co-culture with $CD68^+$ dTHP1 prompted an intracellular production of COX-2 in Sh1b cells. The intracellular COX-2 expression from dTHP1 decreased when they were treated with conditioned medium from cultured Sh1b cells. Present results are in agreement with studies using co-culture between human mammary cancer cells and macrophages, where it was shown an increased expression of COX-2 in the cancer cells side [97, 98]. This effect represents a significant advance on understanding the possible role of COX-2 in inducing a cancer tolerogenic microenvironment in CMT namely through a cancer associated macrophages immunomodulation.

Overall, the present work demonstrated that, similarly to human breast cancer, also in CMT the inflammatory responses in mammary cancer sites are able to orchestrate hallmark-facilitating programs in tumor microenvironment [99]. These phenomena could be important for prognosis as well as for the development of therapies aiming at redirecting the immune cells actions toward tumor destruction.

Additionally, the apparent parallelism between human breast cancer and CMT regarding the role of immune system in carcinogenesis, together with the fact that dogs develop spontaneous tumors in the context of a natural immune system highlight the dog as a valuable model for studies in human breast cancer immunology.

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