Conrado de Oliveira Gamba

# ESTUDO MOLECULAR DA TRANSIÇÃO EPITELIAL MESENQUIMAL EM CARCINOMAS MICROPAPILARES INVASIVOS DA GLÂNDULA MAMÁRIA CANINA

Universidade Federal de Minas Gerais
Programa de Pós-Graduação em Patologia
Belo Horizonte - MG
Fevereiro de 2016

UNIVERSIDADE FEDERAL DE MINAS GERAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA DA UFMG
FACULDADE DE MEDICINA

TESE DE DOUTORADO

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Tese apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina da Universidade Federal de Minas Gerais como parte dos requisitos para a obtenção do título de doutorado. Área de Concentração: Patologia Investigativa

Orientador: Prof. Dr. Geovanni Dantas Cassali
Co-orientador: Prof. Dr. Enio Ferreira

Belo Horizonte - MG
Faculdade de Medicina - UFMG
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**UNIVERSIDADE FEDERAL DE MINAS GERAIS**

**PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA**

# ATA DA DEFESA DE TESE DO ALUNO
# CONRADO DE OLIVEIRA GAMBA

Realizou-se, no dia 22 de fevereiro de 2016, às 09:00 horas, CAD-1, Sala 301, Campus Pampulha, da Universidade Federal de Minas Gerais, a defesa de tese, intitulada *ESTUDO MOLECULAR DA TRANSIÇÃO EPITELIAL MESENQUIMAL EM CARCINOMAS MICROPAPILARES INVASIVOS DA GLÂNDULA MAMÁRIA CANINA*, apresentada por CONRADO DE OLIVEIRA GAMBA, número de registro 2012667150, graduado no curso de MEDICINA VETERINÁRIA, como requisito parcial para a obtenção do grau de Doutor em PATOLOGIA, à seguinte Comissão Examinadora: Prof. Geovanni Dantas Cassali - Orientador (UFMG), Prof. Victor Piana Andrade (AC Camargo Câncer Center), Prof. Rafael Malagoli Rocha (FM - UNIFESP), Profa. Geórgia Modé Magalhães (UNIFRAN), Dra. Michele Ângela Rodrigues (UFMG).

A Comissão considerou a tese:

(X) Aprovada

( ) Reprovada

Finalizados os trabalhos, lavrei a presente ata que, lida e aprovada, vai assinada por mim e pelos membros da Comissão.
Belo Horizonte, 22 de fevereiro de 2016.

Prof. Geovanni Dantas Cassali ( Doutor )

Prof. Victor Piana Andrade ( Doutor )

Prof. Rafael Malagoli Rocha ( Doutor )

Profa. Geórgia Modé Magalhães ( Doutora )

Prof(a). Michele Ângela Rodrigues ( Doutora )

UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA

UFMG

# FOLHA DE APROVAÇÃO

**ESTUDO MOLECULAR DA TRANSIÇÃO EPITELIAL MESENQUIMAL EM CARCINOMAS MICROPAPILARES INVASIVOS DA GLÂNDULA MAMÁRIA CANINA**

## CONRADO DE OLIVEIRA GAMBA

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em PATOLOGIA, como requisito para obtenção do grau de Doutor em PATOLOGIA, área de concentração PATOLOGIA INVESTIGATIVA.

Aprovada em 22 de fevereiro de 2016, pela banca constituída pelos membros:

Prof. Geovanni Dantas Cassali - Orientador
UFMG

Prof. Victor Piana Andrade
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Prof. Rafael Malagoli Rocha
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Profa. Geórgia Modé Magalhães
UNIFRAN

Dra. Michele Angela Rodrigues
UFMG

Belo Horizonte, 22 de fevereiro de 2016.

*“A mente que se abre a uma idéia,*

*jamais voltará ao seu tamanho original”*

***Albert Einstein***

DEDICATÓRIA

*Aos meus pais Enizaldo e Enéia*

*Aos meus irmãos Estêvão e Thiago*

*A minha amada Elis*

AGRADECIMENTOS

Aos meus maravilhosos pais Enizaldo e Enéia por terem me dado a vida. Pelo amor, carinho e exemplo de vida. Sem eles não teria conseguido nada.

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LISTA DE ABREVIATURAS

$Ca^{2+}$ - Cálcio

CMI - Carcinoma Micropapilar Invasivo

SNAIL - Zinc Finger Factor Snail

TEM - Transição Epitelial Mesenquimal

ZEB1 - Zinc Finger E-box Binding Homeobox-1

ZEB2 - Zinc Finger E-box Binding Homeobox-2

RESUMO

A Transição Epitelial mesenquimal (TEM) é um processo pelo qual as células neoplásicas epiteliais perdem a expressão de E-caderina, mediada pelos fatores de transcrição SNAIL, ZEB1, ZEB2 e TWIST, subsequentemente adquirindo um fenótipo mesenquimal com aumento do comportamento migratório. Alguns fatores de transcrição como ZEB1 e ZEB2, tem apresentado relação com o prognóstico e metastatização do câncer em humanos nas mais diferentes localizações. Desta forma, o objetivo deste estudo foi avaliar a expressão proteica de moléculas envolvidas com a TEM, como a E-caderina e seus repressores transcricionais, e a implicação prognóstica de ZEB1 e ZEB2 em carcinomas micropapilares invasivos (CMI), neoplasias agressivas e metastatizantes da glândula mamária canina. Por meio da técnica de imuno-histoquímica observou-se que os CMI apresentam diminuição da expressão de E-caderina em áreas carcinomatosas *in situ* em relação às áreas invasoras demonstrando tendências para um aumento de sua expressão em metástases para linfonodo. Observou-se também uma redução gradativa da expressão de SNAIL de áreas carcinomatosas *in situ* para áreas invasoras, e focos metastáticos em linfonodos. Porém, na análise de ZEB1 observou-se aumento da imunoexpressão de áreas *in situ* e invasoras para metástases em linfonodos. A expressão de ZEB2 foi observada em 52%, 38% e 33% das áreas *in situ*, áreas invasoras e metástases para linfonodo, respectivamente. A expressão de TWIST foi observada em 41%, 28% e 0% das áreas *in situ*, áreas invasoras e metástases para linfonodo, respectivamente. Em áreas invasivas, a diminuição da expressão de E-caderina correlacionou significantemente com o aumento da expressão de SNAIL e TWIST. Adicionalmente, em componentes infiltrativos dos CMI, células neoplásicas E-caderina-negativas e SNAIL-positivas foram observadas por imunofluorescência. No estudo de comportamento prognóstico tumoral, a especifidade para os anticorpos anti-ZEB1 e -ZEB2 foi confirmada pela técnica de Western Blot. A positividade para ZEB2 citoplasmático apresentou relação direta com a menor sobrevida global de cães portadores de CMI; e foi mais frequentemente observada em áreas *in situ* quando comparadas às áreas invasoras desta neoplasia. A positividade para ZEB1 foi associada com a diminuição do grau histológico. Diante desses resultados, podemos concluir que a progressão metastática dos CMI caninos está relacionada à perda da expressão de E-caderina, induzida pelo fator de transcrição SNAIL, e pela expressão de ZEB2. A proteína ZEB2 aparenta ser um fator prognóstico importante em cadelas portadoras de CMI mamários.

**Palavras-chave:** Cão. Carcinoma mamário. Imuno-histoquímica. Imunofluorescência. Transição epitelio-mesenquimal. Fatores de transcrição.

ABSTRACT

The epithelial mesenchymal transition (EMT) is a process whereby neoplastic epithelial cells lost E-cadherin expression, mediated by the transcription factors SNAIL, ZEB1, ZEB2 and TWIST, afterwards acquiring a mesenchymal phenotype and increasing its migratory behavior. Some transcription factors, such as ZEB1 and ZEB2, have showed relationship with the prognosis and metastatisation process in different locations of the human cancer. Hence, the aim of this study was to evaluate protein expression of molecules related to EMT, such as E-cadherin and its transcriptional repressors, and the prognostic value of ZEB1 and ZEB2 in invasive micropapillary carcinomas (IMPC), which are aggressive and metastasising neoplasms of the canine mammary gland. Through immunohistochemistry we have observed that E-cadherin expression decrease from carcinoma *in situ* to invasive progression and was likely to increase in lymph node metastasis of IMPCs. SNAIL expression decreased gradually from carcinoma *in situ* to invasive areas and to metastatic focus in lymph nodes. However, in ZEB1 analysis we have observed increase of immunoexpression from *in situ* and invasive areas to lymph node metastasis. ZEB2 expression was observed in 52%, 38% and 33% of carcinoma *in situ* areas, invasive areas and lymph node metastases, respectively. TWIST expression was observed in 41%, 28% and 0% of carcinoma *in situ* areas, invasive areas and lymph node metastases, respectively. In invasive areas, E-cadherin downregulation correlated significantly with SNAIL and TWIST upregulation. Additionally, in infiltrating components of IMPCs, E-cadherin-negative SNAIL-positive neoplastic epithelial cells were observed by immunofluorescence. In the study related to tumour prognostic behavior the specificity for anti-ZEB1 and –ZEB2 antibodies was confirmed by western blot technique. The positivity for cytoplasmic ZEB2 showed direct relation to poor overall survival of dogs showing IMPC; and was more frequently observed in *in situ* areas than in invasive areas of this neoplasm. ZEB1 positivity was associated with a decrease of histological grade. Thus, we concluded that the metastatic progression of canine IMPC is related to the loss of E-cadherin expression, induced by the transcription factor SNAIL, and for ZEB2 expression. The ZEB2 protein appears to be an important prognostic factor in bitches with mammary IMPCs.

**Keywords:** Dog. Mammary Carcinoma. Immunohistochemistry. Immunofluorescence. Epithelial-mesenchymal transition. Transcription factors.

# 1. INTRODUÇÃO

A Transição Epitelial mesenquimal (TEM) é um processo reversível pelo qual as células de origem epitelial perdem características epiteliais e polaridade, subsequentemente adquirindo um fenótipo mesenquimal com aumento do comportamento migratório (SARRIÓ et al., 2008). Recentemente estudos tem descrito um envolvimento da TEM com a progressão, invasão e disseminação metastática do câncer (THIERY et al., 2009). Molecularmente, a TEM caracteriza-se pela perda da expressão de proteínas de adesão de células epiteliais, como a E-caderina, e aquisição da expressão de marcadores mesenquimais, como a vimentina (GREGORY et al., 2008). A perda da expressão de E-caderina se dá em decorrência de alterações em seu promotor por silenciamento epigenético através de hipermetilação e/ou repressão transcricional mediada por fatores de transcrição, dentre eles o Zinc Finger Factor Snail (SNAIL), TWIST, Zinc Finger E-box Binding Homeobox-1 (ZEB1) e Zinc Finger E-box Binding Homeobox-2 (ZEB2) (BARANWAL; ALAHARI, 2009; COWIN; ROWLANDS; HATSELL, 2005). Além de exercerem a repressão transcricional, os fatores de transcrição ZEB1 e ZEB2 têm sido recentemente relacionados ao processo de progressão maligna e à determinação de pior prognóstico do câncer em humanos nas mais diferentes localizações, incluindo a glândula mamária (KARIHTALA et al., 2013; LEMMA et al., 2013; MAEDA et al., 2005; VANDEWALLE; VAN ROY; BERX, 2009). Neoplasias malignas de ocorrência espontânea na espécie canina tem sido consideradas um modelo adequado para a elucidação de aspectos terapêuticos e biológicos do câncer na espécie humana. A glândula mamária da cadela apresenta um número significativo de lesões patológicas similares àquelas da mama da mulher (CASSALI et al., 2007; VAIL; MACEWEN, 2000). Cassali et al. (1999, 2002) descreveram um subtipo de carcinoma com aspectos histológicos semelhantes ao do carcinoma micropapilar invasivo (CMI) da mama humana. Histopatologicamente essa neoplasia revela inúmeros espaços pseudocísticos, semelhantes a vasos linfáticos, apresentando em seu interior pequenos grupamentos de células epiteliais com ausência de estroma fibrovascular no seu centro, e com a polaridade celular invertida. Na espécie canina, assim como em humanos, o CMI apresenta altas taxas de invasão linfática peritumoral e de metástase para linfonodos regionais sendo por isso considerado um subtipo histológico agressivo e de pior prognóstico (GAMBA et al., 2013; LUNA-MORE et al., 1994).

O CMI canino demonstra comportamento biológico extremamente agressivo, o que permite a utilização deste subtipo como modelo espontâneo para o estudo de novas estratégias terapêuticas e compreensão da evolução e patogênese do câncer de mama em estadiamento avançado. Diante do exposto, a utilização deste subtipo tumoral pode fornecer informações relevantes sobre os mecanismos de regulação da TEM e as implicações prognósticas da expressão de moléculas relacionadas com a TEM em neoplasias mamárias.

Diante disso, no presente estudo avaliou-se a expressão proteica de moléculas relacionadas com a TEM, como a E-caderina e seus repressores transcricionais, e a implicação prognóstica dos fatores de transcrição ZEB1 e ZEB2 em carcinomas micropapilares invasivos mamários caninos.

# 2. REVISÃO DE LITERATURA

## 2.1 E-CADERINA: UMA MOLÉCULA DE ADESÃO CELULAR COM VALOR PROGNÓSTICO EM NEOPLASIAS

A adesão e manutenção da polaridade entre células de vertebrados geralmente é mediada por moléculas conhecidas como junções de adesão, junções de oclusão e desmossomos. Dentre os principais componentes das junções de adesão e desmossomos pode-se destacar as caderinas que consistem em uma grande família de moléculas transmembranares que medeiam adesão intercelular $Ca^{2+}$ (cálcio)-dependente funcionando como proteínas essenciais para a morfogênese de uma variedade de órgãos (CAVALLARO; CHRISTOFORI, 2004; HAN et al., 1999). A superfamília das caderinas é composta por caderinas clássicas, não clássicas e, as mais recentemente descobertas, protocaderinas. Dentre as caderinas do tipo clássico pode-se destacar as caderinas epiteliais (E-caderinas), placentais (P-caderinas) e neuronais (N-caderinas) (PAREDES et al., 2012).

As E-caderinas consistem em um grupo de glicoproteínas transmembranares que medeiam, em condições normais, adesões $Ca^{2+}$ dependentes, entre células epiteliais, mediada por interações homofílicas (PARK et al., 2007). Em neoplasias, a perda da expressão desta proteína tem sido constantemente relacionada a características clinicopatológicas desfavoráveis (HEIMANN et al., 2000; OKA et al., 1992; RAKHA et al., 2005; ZHENG; DU; JIANG, 2015), sobrevida global curta (GURIEC et al., 1995; HEIMANN et al., 2000;

LIU et al., 2013; PARK et al., 2007; RAKHA et al., 2005; SIITONEN et al., 1996; YAN et al., 2014; ZHENG; DU; JIANG, 2015) e metástases para linfonodos e/ou à distância (HUNT et al., 1997; OKA et al., 1992, 1993; RAKHA et al., 2005; UMBAS et al., 1992); além disso, a diminuição da expressão desta proteína também tem sido relacionada com eventos ligados à progressão neoplásica como a Transição Epithelial-Mesenquimal (TEM) (MOYRET-LALLE; RUIZ; PUISIEUX, 2014).

## 2.2 TRANSIÇÃO EPITELIAL MESENQUIMAL

A TEM é um processo de transdiferenciação em que células epiteliais polarizadas transformam-se em células com fenótipo mesenquimal fibroblastóide apresentando aumento de motilidade (DRASIN; ROBIN; FORD, 2011; HUBER; KRAUT; BEUG, 2005). Em mamíferos, a ocorrência deste fenômeno tem sido descrita tanto em processos fisiológicos quanto em processos patológicos, sendo ele classificado em tipo I, II e III.

A TEM tipo I está relacionada a eventos fisiológicos como o desenvolvimento normal de invertebrados; a TEM tipo II está diretamente ligada aos processos fisiológicos de reparo tecidual e patológicos de fibrose parenquimatosa; a TEM tipo III, também conhecida como TEM oncogênica, é mais recente e relaciona-se à progressão e metástase de neoplasias malignas (DRASIN; ROBIN; FORD, 2011; THIERY et al., 2009). Tanto em processos patológicos quanto em processos fisiológicos, levando em consideração o aspecto molecular, as células que sofrem a TEM apresentam perda da expressão de marcadores epiteliais, como a E-caderina e aquisição de moléculas mesenquimais como a vimentina, fibronectina e a N-caderina (DRASIN; ROBIN; FORD, 2011).

A ocorrência da TEM oncogênica tem sido amplamente investigada em neoplasias pela utilização de modelos in *vitro* e de modelos murinos *in vivo,* em contrapartida investigações deste evento em modelos espontâneos de neoplasias são raramente apresentadas na literatura.

Tanto os estudos *in vivo* quanto os *in vitro* tem contribuído amplamente para a elucidação das vias e moléculas responsáveis pela ocorrência da TEM no câncer. A maior parte deles é direcionado para a ocorrência da perda da expressão de E-caderina que tem sido relacionada com mecanismos epigenéticos como a metilação das sequências promotoras do gene CDH1

(responsável pela expressão de E-caderina), modificações em histonas (metilação, acetilação e ubiquitinação), ação de microRNAs (moléculas de RNA que medeiam o silenciamento pós-transcricional de genes-alvo) e repressões transcricionais mediadas por um grupo restrito de fatores de transcrição (LOMBAERTS et al., 2006; PENG et al., 2014). Dentro deste grupo estão as moléculas da família Zinc Finger Transcription Factors relacionados ao Snail (SNAIL1), da família dos Zinc Finger E-Box Binding Homeobox [Zinc Finger E-Box Binding Homeobox-1 (ZEB1/δEF-1/TCF-8/AREB6) e Zinc Finger E-Box Binding Homeobox-2 (ZEB2/SIP1/ZFXH1B)] e da família dos Basic Helix-Loop-Helix (TWIST), que têm como alvo E-boxes específicos localizados na região proximal da sequência promotora do gene CDH1 (DRASIN; ROBIN; FORD, 2011; PEINADO; OLMEDA; CANO, 2007; PENG et al., 2014). Além do papel na repressão transcricional, alguns fatores de transcrição, tem apresentado relação com o prognóstico, progressão e metastatização do câncer nas mais diferentes localizações.

## 2.3 RELAÇÃO DOS FATORES DE TRANSCRIÇÃO SNAIL, TWIST, ZEB1 E ZEB2 COM O PROGNÓSTICO, PROGRESSÃO E METASTATIZAÇÃO DE NEOPLASIAS

O fator de transcrição SNAIL foi inicialmente relacionado com propriedades invasivas e metastáticas de linhagens de células de humanos e camundongos derivadas de tecidos como a pele, mama, cólon, mucosa oral e melanoma (BATLLE et al., 2000; CANO et al., 2000; POSER et al., 2001; YOKOYAMA et al., 2001). A partir destes achados, pesquisas relacionadas à implicação prognóstica desta proteína em neoplasias primárias humanas tem sido conduzidas, associando a expressão da mesma com perda da diferenciação celular e/ou metástase para linfonodos nas mais diferentes localizações, incluindo a glândula mamária (BLANCO et al., 2002; GOU et al., 2014; POBLETE et al., 2014; ZHAO et al., 2013). Além disso, esta molécula também tem sido relacionada com sobrevida global curta de pacientes portadores de mesoteliomas malignos pleurais e de neoplasias mamárias positivas para receptor de estrógeno (KOBAYASHI et al., 2013; VAN NES et al., 2012). Em cães IM et al., (2012) descreveram uma relação da positividade da molécula com aumento do grau histológico e invasão linfática em neoplasias mamárias.

Da mesma forma que SNAIL, a expressão de TWIST tem sido associada com um pior prognóstico do câncer em humanos, correlacionando-se com alto grau histológico, estadio

avançado e incremento no comportamento metastático de neoplasias malignas da mama, bexiga urinária, da próstata, do cérebro e da tireóide (CALANGIU et al., 2014; KWOK et al., 2005; LIM et al., 2015; NORDFORS et al., 2015; ZHANG et al., 2007); além disso, a expressão da proteína também tem sido associada a uma menor sobrevida global de pacientes portadores de neoplasias malignas cervicais, pulmonares e mamárias (GRZEGRZOLKA et al., 2015; SHIBATA et al., 2008; SOINI et al., 2011; ZENG et al., 2015). Na espécie felina, BAPTISTA et al. (2012) descreveram maior expressão gênica de TWIST em carcinomas mamários em relação às lesões hiperplásicas.

A expressão de ZEB1 tem sido correlacionada com neoplasias endometriais agressivas como os adenorcarcinomas de alto grau, leiomiossarcomas, carcinomas papilares serosos e carcinossarcomas (SPOELSTRA et al., 2006). Uma correlação direta entre imunoreatividade para ZEB1 e o sistema de graduação de Gleason tem sido reportada em tumores da próstata humana (GRAHAM et al., 2008). Em linfomas difusos de células B, carcinomas pleomórficos do pulmão, adenocarcinomas ductais pancreáticos, carcinomas hepatocelulares, carcinomas gástricos e coloretais, alta expressão da proteína ZEB1 tem sido associada com sobrevida curta de seres humanos (BRONSERT et al., 2014; HASHIGUCHI et al., 2013; LEMMA et al., 2013; MIYAHARA et al., 2015; MURAI et al., 2014; OKUGAWA et al., 2012; ZHANG et al., 2013; ZHOU et al., 2012). Em relação aos tumores mamários, positividade para ZEB1 tem sido correlacionada com alto grau histológico e ausência de diferenciação celular (AIGNER et al., 2007; KARIHTALA et al., 2013). Adicionalmente, uma associação de ZEB1 com presença de metástase foram reportadas em osteossarcomas, carcinomas hepatocelulares, carcinomas gástricos e coloretais (OKUGAWA et al., 2012; SAYAN et al., 2009; SHEN et al., 2012; ZHANG et al., 2013; ZHOU et al., 2012).

Em relação a ZEB2, a expressão elevada desta molécula tem sido relacionada com a transformação maligna de neoplasias ovarianas e uroteliais; e com alto grau histológico e estadio avançado de carcinomas coloretais e adenocarcinomas gástricos (DAI et al., 2012; KAHLERT et al., 2011; LEE et al., 2013; YOSHIDA et al., 2009). Adicionalmente, a positividade para ZEB2 está relacionado a uma menor sobrevida em paciente com carcinomas coloretais, carcinomas serosos ovarianos, carcinomas de células transicionais, adenocarcinomas gástricos, carcinomas de células escamosas da cavidade oral e faringeal,

carcinoma de células renais, adenocarcinomas ductais pancreáticos e câncer de pulmão (DAI et al., 2012; FANG et al., 2013; JOUPPILA-MÄTTÖ et al., 2015; KAHLERT et al., 2011; KURAHARA et al., 2012; MAEDA et al., 2005; MIURA et al., 2009; SAYAN et al., 2009; YOSHIHARA et al., 2009). Em neoplasias mamárias tendências para sobrevida curta foram recentemente relacionadas a pacientes portadores de câncer de mama (KARIHTALA et al., 2013). Interessantemente a expressão desta molécula também tem sido relacionada com o surgimento de metástases linfáticas regionais em casos de neoplasias gástricas, pancreáticas e orofaríngeas (DAI et al., 2012; JOUPPILA-MÄTTÖ et al., 2015; KURAHARA et al., 2012; SAKAMOTO et al., 2012).

## 2.4 MODELOS PARA ESTUDO DO CÂNCER

Visando a elucidação das principais vias e moléculas relacionadas ao câncer, sua progressão e prognóstico, inúmeros modelos tem sido aplicados em pesquisas direcionadas para a oncologia. Dentre eles destacam-se os modelos *in vitro,* que consistem principalmente na utilização de cultivos de linhagens de células neoplásicas, e modelos *in vivo* que baseiam-se na utilização de neoplasias induzidas em animais de laboratório (roedores) e neoplasias espontâneas ocorrentes em animais, principalmente na espécie canina. Comparativamente, os resultados obtidos com modelos *in vivo* têm sido considerados mais representativos do que em modelos *in vitro*, visto que reproduzem de forma mais fidedigna as condições ambientais do surgimento de uma neoplasia que, neste modelo, apresenta influência dos componentes estromais e do infiltrado inflamatório do hospedeiro (PINHO et al., 2012).

Em relação aos modelos *in vivo* para o estudo do câncer*,* algumas vantagens são apontadas para a utilização dos modelos espontâneos caninos quando comparados aos modelos induzidos: primeiramente a espécie canina apresenta inúmeras similaridades anatômicas e fisiológicas com a espécie humana; além disso, revela maior homologia genômica com seres humanos do que os animais de laboratório (roedores); e por fim, diferentemente dos animais de laboratório, caninos apresentam elevada diversidade fenotípica compatível com que é observado em seres humanos acometidos pelo câncer (KHANNA et al., 2006; LANGDON, 2012; PINHO et al., 2012; RANIERI et al., 2013; ROWELL; MCCARTHY; ALVAREZ, 2011; SHEARIN; OSTRANDER, 2010). Adicionalmente, estudos comparativos tem relatado na espécie canina a ocorrência de neoplasias análogas às ocorrentes em humanos

(MACEWEN, 1990; PINHO et al., 2012). Dentre as similaridades pode-se destacar a aparência histopatológica, perfil de expressão gênica, comportamento biológico e resposta às terapias convencionais (KHANNA et al., 2006; MACEWEN, 1990; WAGNER et al., 2013). As neoplasias caninas mais frequentemente citadas em estudos comparativos são os osteossarcomas (ANGSTADT et al., 2011, 2012; BRODEY, 1979; DE MARIA et al., 2009; FENGER; LONDON; KISSEBERTH, 2014; KOBAYASHI et al., 2013; MORELLO; MARTANO; BURACCO, 2011; MUELLER; FUCHS; KASER-HOTZ, 2007), linfomas (COMAZZI; GUSCETTI; MARCONATO, 2014; ITO; FRANTZ; MODIANO, 2014; MARCONATO; GELAIN; COMAZZI, 2013; MODIANO et al., 2007; PATRUNO et al., 2006) e neoplasias mamárias (KUMARAGURUPARAN et al., 2006; LANGDON, 2012; PINHO et al., 2012).

Em relação ao modelo comparativo canino de neoplasias mamárias, inúmeras similaridades entre as espécies humana e canina tem sido relatadas: em ambas espécies esses tumores são muito frequentes, estão relacionados à etiologia hormonal, possuem fatores de risco semelhantes (tratamento com progestágenos, idade avançada e obesidade) e compartilham características morfológicas, biológicas e moleculares (KLOPFLEISCH et al., 2010; QUEIROGA et al., 2011; RIVERA; VON EULER, 2011; STRANDBERG; GOODMAN, 1974; UVA et al., 2009). Lesões muito bem consolidadas na glândula mamária da espécie humana tem sido descritas em cães, dentre elas destacam-se as lesões pré-neoplásicas, como hiperplasias e lesões de células colunares (ANTUOFERMO et al., 2007; FERREIRA et al., 2010, 2012; MOUSER et al., 2010), carcinomas lobulares pleomórficos (CASSALI et al., 2002; SALGADO et al., 2012), carcinomas com diferenciação sebácea (CHANG et al., 2007; GRANDI et al., 2011), carcinomas micropapilares invasivos (CASSALI et al., 1999, 2002; GAMA; ALVES; SCHMITT, 2008; GAMBA et al., 2013; SALGADO et al., 2013) e tumores filodes (ARAÚJO et al., 1996; SHAHZAMANI; TAKHTFOOLADI; DANESHI, 2013).

## 2.5 MODELO COMPARATIVO ESPONTÂNEO CANINO DE CARCINOMA MICROPAPILAR INVASIVO DA GLÂNDULA MAMÁRIA

Os carcinomas micropapilares invasivos (CMI) da glândula mamária são neoplasias de rara ocorrência na espécie humana representando entre 1,7 e 7% dos casos de câncer de mama na mulher (LUNA-MORE et al., 1996; LUNA-MORÉ et al., 2000; NASSAR et al., 2001;

PATERAKOS et al., 1999; WALSH; BLEIWEISS, 2001). Apesar de sua baixa frequência, esta entidade tem elevada importância diagnóstica, visto que está diretamente ligada a altas taxas de invasão linfática (linfotropismo) e de metástase para linfonodos regionais, além sobrevida global curta (LUNA-MORÉ et al., 2000; SIRIAUNKGUL; TAVASSOLI, 1993). Histopatologicamente CMI apresentam inúmeras áreas de invasão estromal, embebidas em estroma espongiforme, caracterizadas por formações císticas revestidas por células fusiformes achatadas, contendo em seu interior grupamentos de células neoplásicas epiteliais sem estroma fibrovascular central, que por seu aspecto morfológico são conhecidos como "vegetações morulares". As células neoplásicas apresentam-se arredondadas a ovaladas, com citoplasma eosinofílico a finamente granular de tamanho variável e atipia nuclear variável. As áreas de invasão podem apresentar-se associadas a áreas carcinomatosas *in situ* que geralmente revelam padrão micropapilar. Outros padrões morfológicos de áreas carcinomatosas *in situ* também podem ser observados, dentre eles os padrões cribriforme, comedo e papilar (LUNA-MORE et al., 1994; MIDDLETON et al., 1999; SIRIAUNKGUL; TAVASSOLI, 1993). Adicionalmente CMI podem apresentar-se associados a outros subtipos de carcinomas, sendo assim considerados mistos/combinados. Dentre os padrões morfológicos de carcinomas mais comumente observados destacam-se carcinomas ductais sem especificação, mucinoso-colóide carcinoma, carcinoma papilar, carcinoma tubular (LUNA-MORÉ et al., 2000; MIDDLETON et al., 1999).

Inúmeros estudos moleculares tem sido conduzidos para elucidação do comportamento biológico agressivo dos carcinomas micropapilares. Dentre as alterações encontradas pode-se destacar a imunopositividade para HER2 (PATERAKOS et al., 1999) e receptores hormonais (receptor de estrógeno e progesterona) (LUNA-MORÉ et al., 2000; WALSH; BLEIWEISS, 2001), perda de heterozigozidade no loco 17p13.1 (p53) (MIDDLETON et al., 1999) e perda de material genético do cromossomo 8p (THOR et al., 2002).

Em cães os CMI espontâneos têm sido descritos na glândula mamária, apresentando semelhanças citológicas, histopatológicas, imuno-histoquímicas e de comportamento biológico com os CMI humanos (CASSALI et al., 1999, 2002, 2015; GAMA; ALVES; SCHMITT, 2008; SALGADO et al., 2013). A presença da neoplasia em cadelas parece associar-se com altas taxas de metástase para linfonodos e sobrevida global curta, além disso,

apresentando alto grau histológico, altas taxas de invasão vascular, positividade para receptor de estrógeno e progesterona, expressão de p53, alto índice proliferativo (avaliado pela expressão de MIB-1) e expressão nuclear de EGFR (GAMBA et al., 2013; RODRIGUES et al., 2015; SALGADO et al., 2014). Diante das inúmeras semelhanças com sua variante humana, CMI caninos parecem apresentar alto potencial como modelo espontâneo para elucidação do comportamento biológico e resposta terapêutica desta importante neoplasia que acomete as mulheres.

## 3. JUSTIFICATIVA

O CMI canino demonstra comportamento biológico extremamente agressivo, o que permite a utilização deste subtipo como modelo espontâneo para o estudo de novas estratégias terapêuticas e compreensão da evolução e patogênese do câncer de mama em estadiamento avançado. Diante do exposto, a utilização deste subtipo tumoral pode fornecer informações relevantes sobre os mecanismos de regulação da TEM e as implicações prognósticas da expressão de moléculas relacionadas com a TEM em neoplasias mamárias.

## 4. HIPÓTESE

CMI mamários caninos, devido ao seu comportamento metastatizante, podem revelar eventos relacionados com transição epitelial mesenquimal, como a perda da expressão de E-caderina, relacionados à ação de repressores transcricionais como o TWIST, SNAIL, ZEB1 e ZEB2, e consequentemente a um prognóstico desfavorável em cães.

## 5. OBJETIVOS

### 5.1 OBJETIVO GERAL

Avaliar a expressão proteica de moléculas envolvidas com a transição epitelial-mesenquimal, E-caderina e seus repressores transcricionais (ZEB1, ZEB2, SNAIL e TWIST), e a implicação prognóstica de ZEB1 e ZEB2 em carcinomas micropapilares invasivos mamários caninos.

### 5.2 OBJETIVOS ESPECÍFICOS

- Caracterizar a expressão proteica de E-caderina e de seus repressores transcricionais (ZEB1, ZEB2, SNAIL e TWIST) em áreas carcinomatosas *in situ,* áreas invasoras e em metástases linfáticas de carcinomas micropapilares da glândula mamária canina;

- Correlacionar a expressão proteica de E-caderina com a expressão de seus repressores transcricionais (ZEB1, ZEB2, SNAIL e TWIST) em áreas carcinomatosas *in situ,* áreas invasoras e em metástases linfáticas de carcinomas micropapilares da glândula mamária canina;

- Investigar a associação da imunoexpressão de ZEB1 e ZEB2 com parâmetros clínico-patológicos e sobrevida global de CMI mamários caninos.

## 5.3 MATERIAL E MÉTODOS, RESULTADOS E DISCUSSÃO

Estes tópicos serão apresentados na forma de artigos científicos.

### 5.3.1 ARTIGO 1

# ZEB2 and ZEB1 expression in a spontaneous canine model of invasive micropapillary carcinoma of the mammary gland

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# ZEB2 and ZEB1 expression in a spontaneous canine model of invasive micropapillary carcinoma of the mammary gland

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ABSTRACT

ZEB1 and ZEB2 have been recently related to cancer prognosis. We investigated their expression and its association with clinicopathological parameters and overall survival in invasive micropapillary carcinoma (IMPC), which is a metastasising neoplasm of the canine mammary gland. Immunohistochemical evaluation showed nuclear and cytoplasmic staining for ZEB2 and nuclear staining for ZEB1. 'In situ' areas presented higher positivity for cytoplasmic ZEB2 than invasive areas of IMPC did ($p = 0.03$). ZEB1 positivity was associated with a low histological grade ($p = 0.01$). A shorter overall survival rate was observed in IMPCs that were positive for cytoplasmic ZEB2 ($p = 0.04$). Antibodies specificity in canine species was confirmed by western blot. Our results indicated that cytoplasmic ZEB2 appears to be an important factor in the early stages of malignancy and predicts a poor overall survival rate for IMPC in this canine mammary cancer model. ZEB1 downregulation appears to be associated with the dedifferentiation process of IMPC.


## 1. Introduction

ZEB2 (ZEB2/SIP1, zinc finger E-box-binding homeobox 2) and ZEB1 (ZEB1/dEF1, zinc finger E-box-binding homeobox 1) are members of the ZEB family, which are essential participants in the normal embryonic development of vertebrates. Additionally, these proteins are involved in pathological contexts, such as syndromic malformations (because of mutations in the ZEB-encoding genes) and the epithelial–mesenchymal transition (EMT) process in neoplasms (Vandewalle et al., 2009). These molecules are increasingly considered to be important contributors to the process of malignant progression and to cancer prognosis (Lemma et al., 2013; Maeda et al., 2005; Vandewalle et al., 2009).

In human species, ZEB2/SIP1 has been related to tumour progression from benign to borderline to malignant ovarian tumours (Yoshida et al., 2009). This molecule has been associated with poor prognosis oral squamous cell carcinomas and bladder cancer (Maeda et al., 2005; Sayan et al., 2009). Breast cancer patients who are positive for ZEB2 trend towards poorer overall survival (Karihtala et al., 2013).

Also in human beings, ZEB1 expression has been correlated with aggressive phenotypes in various histological types of endometrial carcinomas (Spoelstra et al., 2006). A direct correlation between ZEB1 immunoreactivity and the Gleason grade in human prostate tumours has been reported (Graham et al., 2008). In diffuse large B-cell lymphoma, high expression of the ZEB1 protein has been associated with an adverse outcome (Lemma et al., 2013). Concerning mammary tumours of women, ZEB1 positivity has been correlated with a higher histological grade (Karihtala et al., 2013). Moreover, an association of ZEB1 with metastatic disease has been reported in gastric, hepatocellular and colon carcinomas (Sayan et al., 2009).

Naturally occurring malignancies in canines are considered to be suitable models for human cancer therapy and cancer biology (Vail and MacEwen, 2000). Canine mammary glands can bear significant pathological lesions that are similar to those of the human breast (Cassali et al., 2007; Vail and MacEwen, 2000). Invasive micropapillary carcinoma (IMPC) in canine mammary glands and its human counterpart represent neoplasms with high rates of stromal and vascular invasion that are frequently associated with lymph node metastasis and a poor prognosis (Gamba et al., 2013).

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The roles of ZEB1 and ZEB2 in tumour progression and prognosis remain poorly understood. To gain insight into this topic, studies that evaluate ZEB1 and ZEB2 expression in infiltrating and metastasising models, such as spontaneous canine IMPC, should be conducted. In this context, the aim of the present study was to investigate ZEB2 and ZEB1 expression and its association with clinicopathological parameters and overall survival in IMPC via a canine mammary cancer model.

## 2. Materials and methods

### 2.1. Case selection and clinical evaluation

Cases of IMPC of the canine mammary gland were selected at the Veterinary School of the Federal University of Minas Gerais, the Federal University of Bahia, and the Laboratory of Comparative Pathology at the Institute of Biological Sciences at the Federal University of Minas Gerais. The cases were staged according to the tumour-node-metastasis (TNM) clinical staging system for canine mammary tumours. This system evaluates tumour size (T1, 0–3 cm; T2, 3–5 cm; T3, >5 cm), involvement of the regional lymph nodes (N0, no metastasis; N1, metastasis), and the presence of distant metastasis (M0, no metastasis; M1, metastasis). The cases were then categorised into five stages: I (T1N0M0), II (T2N0M0), III (T3N0M0), IV (T1,2,3N1M0), and V (T1,2,3N0,1M1) (Owen, 1980).

### 2.2. Histopathological analysis

The tumour specimens were fixed in 10% neutral buffered formalin and embedded in paraffin, and 4-μm-thick histological sections were cut and stained with haematoxylin and eosin. All cases were reviewed and independently re-classified by two veterinary pathologists (COG and GDC) based on histopathological characteristics described by Misdorp et al. (1999) and Cassali et al. (2011, 2014). IMPCs were classified as being of the 'pure' (carcinomas with a ≥75% infiltrating micropapillary pattern) or 'mixed' (carcinomas with a <75% infiltrating micropapillary pattern, associated with other infiltrating carcinomas) subtype (Gamba et al., 2013). The invasive areas were graded according to the Nottingham grading system (Elston and Ellis, 1991, 1998). IMPCs were grouped into low (grade 1) and high (grades 2 and 3) histological grades. The mitotic index (MI) was assessed as the number of mitoses per 10 fields at the tumour periphery, and the tumours were classified as having a low (≤7 mitoses) or high (≥8 mitoses) MI.

### 2.3. Immunohistochemistry and scoring

For the immunohistochemical analysis, consecutive 4-μm-thick sections were mounted on silanised slides, and a peroxidase system was used along with secondary antibodies, which were identified using Advance HRP (Dako North America Inc., Carpinteria, California, USA). Endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution in methyl alcohol. The reagents were applied manually, and immunoreactivity was visualised by incubating the slides with diaminobenzidine (Lab Vision DAB substrate system) for 10 min. The details of the antibodies, dilutions, antigen retrieval procedures, and incubation times of the immunostaining process are shown in Table 1. Sections from ZEB2- and ZEB1-positive canine fibrosarcomas were used as positive controls. Negative controls were assessed using normal serum (Lab Vision Ultra V Block) in the place of the primary antibody when analysing a sample of canine IMPC.

To evaluate tumour progression, an immunohistochemical analysis was performed in 'in situ' (non-invasive) and in invasive areas of IMPC. Cytoplasmic and nuclear staining was evaluated for ZEB2. Only nuclear staining was assessed for ZEB1. ZEB2 cytoplasmic immunoreactivity was scored for its extent and intensity according to the protocol of Karihtala et al. (2013). Staining intensity was graded in the following manner: negative (0), weak (1), moderate (2) or strong (3). Staining extent was rated according to the percentage of positive cells: samples with 0–2% of stained tumour cells were rated as 0, those with 2–10% of stained tumour cells were rated as 1, those with 10–50% of stained tumour cells were rated as 2, and those with >50% of stained tumour cells were rated as 3. The staining intensity and extent were summed, with an overall staining score that varied from 1 to 6. The quantitative and qualitative estimates were combined in the following manner: 0 = negative, 1–2 points = 1, 3–4 points = 2, and 5–6 points = 3. IMPCs were categorised as either 0–1 (negative) or 2–3 (positive). Nuclear ZEB2 and ZEB1 positivity was assessed based on the presence (+) or absence (–) of staining (Geradts et al., 2011; Karihtala et al., 2013).

### 2.4. Western blot analysis

To evaluate anti-ZEB1 and anti-ZEB2 antibody specificity in canine species, western blot analysis was performed using two fresh samples of canine mammary tissues (one normal mammary gland and one tumoural mammary gland). The tumoural mammary gland consisted of a carcinoma sample. Whole-cell extracts were obtained from homogenised samples by using a lysis buffer (1% Triton X-100; 100 mM Tris/HCl, pH 8.0; 10% glycerol; 5 mM EDTA; 200 mM NaCl; 1 mM DTT; 1 mM PMSF; 25 mM NaF; 2.5 μg/ml leupeptin; 5 μg/ml aprotinin; and 1 mM sodium orthovanadate). Lysates were centrifuged at 13,000 g for 10 min at 4 °C and quantified using the Bradford assay reagent from Bio-Rad (Hercules, CA). Extracts (30 μg) were separated by electrophoresis on a denaturing 8% polyacrylamide–sodium dodecyl sulphate gel and transferred onto nitrocellulose membranes, as previously described (De Sousa et al., 2005). The membranes were blocked overnight at 4 °C with phosphate-buffered saline (PBS) containing 5% (w/v) nonfat dry milk and 0.1% Tween-20, washed three times with PBS containing 0.1% Tween-20, and then incubated with specific anti-ZEB1 (1:500; Sigma-Aldrich, St. Louis, MO, USA) and anti-ZEB2 (1:500; Sigma-Aldrich, St. Louis, MO, USA) rabbit polyclonal antibodies in PBS containing 5% (w/v) BSA and 0.1% Tween-20. As a loading control, the membranes were reprobed with anti-β-actin (1:5000; Sigma-Aldrich, St. Louis, MO, USA). After washing, the membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000; Santa Cruz Biotechnology). Immunoreactive bands were visualised using an enhanced chemiluminescence detection system as described by the manufacturer (GE Healthcare, Piscataway, NJ).

**Table 1**
Details of the immunohistochemical staining and the reagents and methods used in the study.

| Target antigen | Clone | Manufacturer | Dilution | AR method | Incubation time (h) | Staining location |
|---|---|---|---|---|---|---|
| ZEB2 | Polyclonal | Sigma-Aldrich | 1:100 | Water bath (98 °C) | 3 | Nuclear/Cytoplasmic |
| ZEB1 | Polyclonal | Sigma-Aldrich | 1:200 | Water bath (98 °C) | 1 | Nuclear |

AR, antigen retrieval; ZEB2, zinc finger E-box-binding homeobox 2; ZEB1, zinc finger E-box-binding homeobox 1.

### 2.5. Statistical analysis

The GraphPad Prism 5.0 software package was used for the statistical analysis. A comparison between the 'in situ' and invasive areas of IMPC was performed using a one-sided chi-square test. An identical test was applied to determine the association between the clinicopathological parameters (tumour size, lymph node metastasis, distant metastasis, clinical staging, histological grading, MI and the subtype of IMPC) and protein expression in IMPC. Only expression in invasive areas was considered in this analysis. Spearman's correlation test was used to estimate the relationship between the staining patterns of the different antibodies used in the 'in situ' and invasive areas.

The overall survival time was defined as the period (in days) between surgery and death due to the tumour. The follow-up period was 720 days. Animals that died from unknown causes or from causes unrelated to the tumour were censored. The prognostic significance of cytoplasmic ZEB2 and nuclear ZEB2 and ZEB1 for determining survival was examined using the Kaplan–Meier method, and statistical significance was examined using a log-rank test. A survival analysis was performed based on staining in the invasive areas of IMPC. Probability values below 0.05 were considered significant for all statistical tests.

## 3. Results

### 3.1. Epidemiological and clinicopathological results for IMPC

In total, 31 IMPCs of the canine mammary gland were obtained. Some epidemiological and clinicopathological data were not available for all 31 IMPCs. The animals ranged from 7 to 15 years old (mean 11.12 ± 1.87) at the time of surgery, and most were purebred bitches (21/30, 70%). Multicentric localisation (16/29, 55%) and a tumour size up to 5 cm were predominant (13/28, 47%). Regional and distant metastases were observed in 96% (23/24) and 25% (5/20) of the dogs, respectively, and stage IV was the most common clinical stage (10/16, 62%). Regarding the histopathological analysis of IMPC, histological grade II (20/31, 64%) and the pure subtype (19/31, 61%) were more frequently observed (Fig. 1A). 'In situ' (non-invasive) ductal carcinomas were observed in 87% (27/31) IMPCs (Fig. 1B). More details of the clinicopathological results for IMPC are presented in Table 2.

### 3.2. Evaluation of ZEB2 and ZEB1 staining in IMPC

For cytoplasmic ZEB2, 'in situ' (non-invasive) areas had higher positivity than invasive areas did ($p = 0.03$) (Fig. 1C and D). Neoplastic epithelial cells showed nuclear positivity for ZEB2, but differences between 'in situ' and invasive areas were not observed. ZEB1 nuclear staining was revealed in tumour-associated stromal fibroblasts and neoplastic epithelial cells in IMPC (Fig. 1E and F). Differences in epithelial cells between the 'in situ' and the invasive areas were not observed. Spearman's test revealed a weak and non-significant correlation between nuclear ZEB2 and cytoplasmic ZEB2 and ZEB1 in both 'in situ' and invasive areas. Table 3 summarises the ZEB2 and ZEB1 immunostaining in 'in situ' and in invasive areas of IMPC.

### 3.3. Association between ZEB2 and ZEB1 staining in invasive areas and clinicopathological features

ZEB1 positivity was associated with a low histological grade ($p = 0.01$). Nuclear and cytoplasmic ZEB2 did not exhibit an association with the clinicopathological features of IMPC. The relationship between the EMT markers and the clinicopathological features is shown in Table 4.

**Table 2**
Clinicopathological features of IMPC in canine mammary glands.

| Characteristic | *N* | IMPC, *n* (%)* |
|---|---|---|
| Breed | 30 | |
| Pure breed | | 21 (70) |
| Cross breed | | 9 (30) |
| Tumour size | 28 | |
| <3 cm | | 9 (32) |
| 3–5 cm | | 6 (21) |
| >5 cm | | 13 (47) |
| Lymph node metastasis[a] | 24 | |
| N0 | | 1 (4) |
| N1 | | 23 (96) |
| Distant metastasis[b] | 20 | |
| M0 | | 15 (75) |
| M1 | | 5 (25) |
| Stage (TNM)[c] | 16 | |
| I | | 1 (6) |
| II | | 0 (0) |
| III | | 1 (6) |
| IV | | 10 (63) |
| V | | 4 (25) |
| 'In situ' areas | 31 | |
| No | | 4 (13) |
| Yes | | 27 (87) |
| Subtype of IMPC[d] | 31 | |
| Pure | | 19 (61) |
| Mixed | | 12 (39) |
| Histological grade[e] | 31 | |
| I | | 8 (26) |
| II | | 20 (64) |
| III | | 3 (10) |

* Some data were not available for all 31 IMPCs.
[a] N0, absence of regional metastasis; N1, regional metastasis.
[b] M0, absence of distant metastasis; M1, distant metastasis.
[c] I (T1N0M0), II (T2N0M0), III (T3N0M0), IV (T1,2,3N1M0), and V (T1,2,3N0,1M1).
[d] 'Pure' subtype: carcinomas with a ≥75% infiltrating micropapillary pattern; 'mixed' subtype: carcinomas with a <75% infiltrating micropapillary pattern, associated with other infiltrating carcinomas.
[e] Histological grading based on that of Elston and Ellis (1991, 1998).

### 3.4. Survival analysis

Survival data were available for 29 cases. In total, 24 dogs (83%) died because of mammary neoplasia, 5 of which were euthanised because of the disease. One dog (6%) died from haemorrhagic diathesis 8 days after surgery, and three dogs (10%) were alive up to 720, 360 or 90 days post-surgery. The median overall survival time was 150 days. The median overall survival time for pure IMPC (120 days) was shorter than that for the mixed subtype (165 days) ($p = 0.67$). Poor overall survival rates were associated with cytoplasmic positivity for ZEB2 ($p = 0.04$) (Fig. 2). Differences between

**Table 3**
Immunohistochemical analysis of ZEB2 (cytoplasmic and nuclear staining) and ZEB1 in canine mammary IMPCs.

| Antigen | IMPC, *n* (%) | | |
|---|---|---|---|
| | 'In situ' | Invasive | *p* value |
| ZEB2 (Cyt) | 27 (100) | 31 (100) | **0.03*** |
| + | 13 (48) | 8 (26) | |
| – | 14 (52) | 23 (74) | |
| ZEB2 (Nu) | 27 (100) | 31 (100) | 0.27 |
| + | 6 (22) | 5 (16) | |
| – | 21 (78) | 26 (84) | |
| ZEB1[a] | 26 (100) | 30 (100) | 0.27 |
| + | 6 (23) | 5 (17) | |
| – | 20 (77) | 25 (83) | |

IMPC, invasive micropapillary carcinoma; ZEB2, zinc finger E-box-binding homeobox 2; ZEB1, zinc finger E-box-binding homeobox 1; Cyt, cytoplasmic; Nu, nuclear.
* Statistical significance $p < 0.05$.
[a] In one case, an evaluation of ZEB1 staining was not possible.

**Table 4**
Expression of cytoplasmic and nuclear ZEB2 and ZEB1 in IMPCs of the canine mammary gland and their relationship with clinicopathological features.

| Clinicopathological feature | | ZEB1[#] | | | ZEB2 (Cyt) | | | ZEB2 (Nu) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *n* (%) | | | *n* (%) | | | *n* (%) | | |
| | *n* | + | – | *p* | + | – | *p* | + | – | *p* |
| Tumour size[a] | | | | | | | | | | |
| T1 | 9 | 1 (11) | 8 (89) | 0.26 | 1 (11) | 8 (89) | 0.07 | 3 (33) | 6 (67) | 0.32 |
| T2–3 | 19 | 4 (21) | 15 (79) | | 7 (37) | 12 (63) | | 8 (42) | 11 (58) | |
| Lymph node metastasis[b] | | | | | | | | | | |
| N0 | 1 | 0 (0) | 1 (100) | 0.32 | 0 (0) | 1 (100) | 0.23 | 0 (0) | 1 (100) | 0.23 |
| N1 | 23 | 4 (17) | 19 (83) | | 8 (35) | 15 (65) | | 8 (35) | 15 (65) | |
| Distant metastasis[c] | | | | | | | | | | |
| M0 | 15 | 3 (20) | 12 (80) | 0.13 | 5 (33) | 10 (67) | 0.06 | 4 (27) | 11 (73) | 0.38 |
| M1 | 5 | 0 (0) | 5 (100) | | 0 (0) | 5 (100) | | 1 (20) | 4 (80) | |
| Stage (TNM)[d] | | | | | | | | | | |
| I–III | 2 | 0 (0) | 2 (100) | 0.28 | 0 (0) | 2 (100) | 0.15 | 0 (0) | 2 (100) | 0.23 |
| IV–V | 14 | 2 (14) | 12 (86) | | 5 (36) | 9 (64) | | 3 (21) | 11 (79) | |
| Histological grade[e] | | | | | | | | | | |
| G1 | 8 | 3 (43) | 4 (57) | **0.01*** | 2 (25) | 6 (75) | 0.47 | 3 (37) | 5 (63) | 0.44 |
| G2–3 | 23 | 2 (9) | 21 (91) | | 6 (26) | 17 (74) | | 8 (35) | 15 (65) | |
| Mitotic index[f] | | | | | | | | | | |
| Low | 21 | 3 (15) | 17 (85) | 0.36 | 5 (24) | 16 (76) | 0.35 | 6 (29) | 15 (71) | 0.26 |
| High | 10 | 2 (20) | 8 (80) | | 3 (30) | 7 (70) | | 4 (40) | 6 (60) | |
| Subtype of IMPC[g] | | | | | | | | | | |
| Pure | 19 | 3 (17) | 15 (83) | 0.50 | 5 (26) | 14 (74) | 0.46 | 6 (32) | 13 (68) | 0.28 |
| Mixed | 12 | 2 (17) | 10 (83) | | 3 (25) | 9 (75) | | 5 (42) | 7 (58) | |

ZEB2, zinc finger E-box-binding homeobox 2; ZEB1, zinc finger E-box-binding homeobox 1; Cyt: cytoplasmic; Nu: nuclear.

* Statistical significance, $p < 0.05$.

[a] T1 (0–3 cm) and T2–3 (>3 cm).

[b] N0 group (absence of lymph node metastasis) and N1 group (presence of lymph node metastasis).

[c] M0 group (absence of metastasis) and M1 group (presence of metastasis).

[d] I–III (T1,2,3N0M0) and IV–V (T1,2,3N0,1M1).

[e] Histological grading based on that of Elston and Ellis (1991, 1998).

[f] Assessed as the number of mitoses per 10 fields at the tumour periphery (low mitotic index: ≤7 mitoses; high mitotic index: ≥8 mitoses).

[g] 'Pure' subtype: carcinomas with a ≥75% infiltrating micropapillary pattern; 'mixed' subtype: carcinomas with a <75% infiltrating micropapillary pattern, associated with other infiltrating carcinomas (Gamba et al., 2013).

[#] In one case, an evaluation of ZEB1 staining was not possible.

positive and negative cases were not observed for nuclear ZEB2 or ZEB1.

### 3.5. Western blot analysis of ZEB1 and ZEB2

According to the manufacturer of the antibodies, the predicted bands for ZEB1 correspond to 124.1, 122.1, 122.0 and 116.6 kDa and for ZEB2 correspond to 136.4 and 133.8 kDa. In tumour samples, we could detect a specific band for ZEB1 between the molecular weights of 124 and 80 kDa (Fig. 3A), and for ZEB2, a band was detected between the molecular weights of 209 and 124 kDa (Fig. 3B). Other nonspecific bands were also detected for ZEB2 in both normal and tumoural tissues.

## 4. Discussion

The molecules in the ZEB family are normally related to EMT induction. Over the last few years, many reports have linked ZEB1 and/or ZEB2 expression to increased aggressiveness and higher metastatic capacity in many primary human carcinomas (Sánchez-Tilló et al., 2012). To better understand this association, we evaluated ZEB2 and ZEB1 expression in IMPC, which is an infiltrating and metastasising neoplasm of the canine mammary gland. The specificity of the antibodies applied in this study was confirmed by western blot analysis performed using canine mammary samples.

ZEB2 is a transcription factor that typically exhibits nuclear expression in neoplasms (Karihtala et al., 2013; Nam et al., 2014). Notably, ZEB2 cytoplasmic staining has been described in kidney, breast, lung, uterus and colorectal tumours (Kahlert et al., 2011; Oztas et al., 2010). In our series, we observed cytoplasmic ZEB2 staining with higher positivity 'in situ' than in invasive areas of IMPC. These results may indicate a major role of ZEB2 in the early stages of tumour progression. An in vitro study by Kahlert et al. (2011) described the ZEB2 protein as responsible for the migration and invasion of neoplastic cells. Therefore, in IMPC, the higher expression of ZEB2 in 'in situ' areas could be associated with the ability to migrate beyond basal membranes and into the tumoural stroma.

Moreover, our results suggest that positive cytoplasmic ZEB2 in invasive areas of IMPC may be used to predict poor overall survival. In humans, overexpression of cytoplasmic ZEB2 has been associated with poor survival in non-small-cell lung carcinoma (NSCLC), oral squamous cell carcinomas and colorectal carcinomas (Kahlert et al., 2011; Maeda et al., 2005; Miura et al., 2009). In NSCLC, cytoplasmic ZEB2 has been associated with an advanced stage, and according to certain authors, it could be related to the poor overall survival of patients. In our research, all ZEB2-positive samples presented at an advanced stage (IV or V), which could explain the short overall survival of these patients. This study did not reveal an association between cytoplasmic ZEB2 positivity and clinical staging; however, this relationship could be confirmed by evaluating a larger sample of IMPCs.

In the present study, ZEB1 was mainly observed in the nuclei of stromal cells, and tumour cells were stained only occasionally. Low expression of ZEB1 has been described in breast cancer epithelial cells (Karihtala et al., 2013; Soini et al., 2011). High rates of stromal staining at the tumour–host interface have been previously reported in mammary neoplasms, colorectal adenocarcinomas and urinary bladder carcinomas (Aigner et al., 2007; Schulte et al., 2012; Soini et al., 2011). Several of these stained stromal cells could represent tumour cells that have undergone ZEB1/dEF1-dependent EMT (Vandewalle et al., 2009).

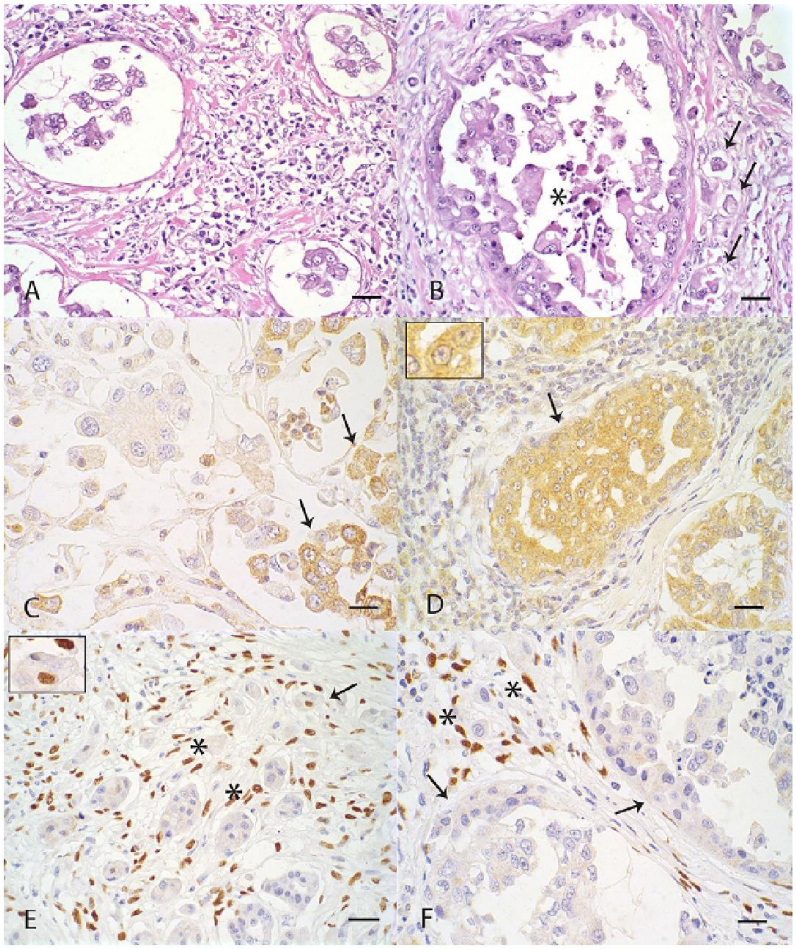


**Fig. 1.** Photomicrographs illustrating the features of invasive and 'in situ' micropapillary carcinomas of the canine mammary gland. (A) Invasive micropapillary areas characterised by neoplastic epithelial cells within cystic spaces (HE stain; scale bar, 30 μm). (B) An 'in situ' micropapillary carcinoma characterised by intra-ductal nests of abnormal malignant cells (asterisk). The lesion is surrounded by a basal membrane. Invasive areas surrounding the 'in situ' areas can be observed (arrows) (HE stain; scale bar, 30 μm). (C) Invasive micropapillary areas exhibiting multifocal, high-intensity cytoplasmic staining for ZEB2 (arrows) (advance HRP peroxidase system and anti-ZEB2; counterstained with Harris's haematoxylin; scale bar, 20 μm). (D) An 'in situ' micropapillary area exhibiting diffuse, high-intensity cytoplasmic staining for ZEB2 (arrow) (inset shows detail) (advance HRP peroxidase system and anti-ZEB2; counterstained with Harris's haematoxylin; scale bar, 30 μm). (E) Nuclear staining for ZEB1 in an invasive area (arrow) (inset shows detail) and in tumour-associated stromal fibroblasts (asterisks) (advance HRP peroxidase system and anti-ZEB1; counterstained with Harris's haematoxylin; scale bar, 30 μm). (F) An absence of ZEB1 staining in the 'in situ' micropapillary areas (arrows). Nuclear staining occurred in tumour-associated stromal fibroblasts (asterisks) (advance HRP peroxidase system and anti-ZEB1; counterstained with Harris's haematoxylin; scale bar, 20 μm).

ZEB1 overexpression in neoplastic epithelial cells has been associated with high histological grades in human invasive breast carcinomas (Karihtala et al., 2013). Notably, in our series, as described by Montserrat et al. (2011), in human ductal carcinomas, IMPC presented an association between ZEB1 negativity in invasive areas and higher histological grades. In breast tumours, a high histological grade has been associated with the dedifferentiation process, regional metastasis and poor overall survival (Elston and Ellis, 1991; Rakha et al., 2008). As in its human counterpart, canine IMPC exhibits a predominance of high histological grades (II and III) and high rates of lymph node metastasis (Marinho et al., 2007; Walsh and Bleiweiss, 2001); therefore, ZEB1 downregulation could be coupled with IMPC dedifferentiation and aggressive behaviour.

In conclusion, our results indicated that cytoplasmic ZEB2 appears to be an important factor in the early stages of malignant progression and predicts a poor overall survival rate for IMPC in a canine

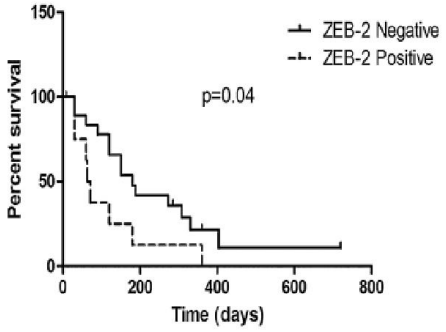


**Fig. 2.** The overall survival curves of 26 cases of IMPC in canine mammary glands. The follow-up period was 770 days. ZEB2-positive IMPCs (median: 67 days) presented lower survival rates than ZEB2-negative IMPCs did (median: 180 days).

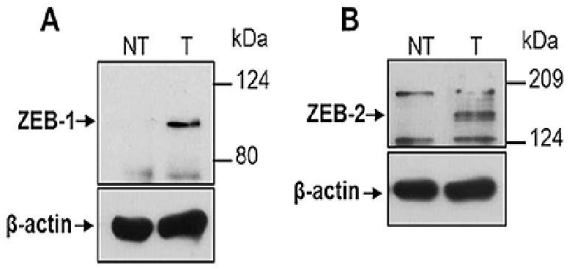


**Fig. 3.** Western blot analysis to detect ZEB1 and ZEB2 in mammary tissues (normal mammary gland, NT; tumoural mammary gland, T) of canine species. Samples were collected and processed for protein extraction for western blot analysis, as described in Section 2. As a loading control, the membranes were reprobed with anti-β-actin. The autoradiographs shown are representative of two experiments for each tissue. Molecular weights are shown to the right of each autoradiograph. In tumour samples, specific bands for ZEB1 and ZEB2 were observed between the molecular weights of 124 and 80 kDa (A) (arrow) and 209 and 124 kDa (B) (arrow), respectively.

mammary cancer model. Moreover, ZEB1 downregulation appears to be associated with the dedifferentiation process of this neoplasm.

## Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could have inappropriately influenced or biased the content of this paper.

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## 5.3.2 ARTIGO 2

# The Relationship Between E-Cadherin and its Transcriptional Repressors in Spontaneously Arising Canine Invasive Micropapillary Mammary Carcinoma

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NEOPLASTIC DISEASE

# The Relationship Between E-Cadherin and its Transcriptional Repressors in Spontaneously Arising Canine Invasive Micropapillary Mammary Carcinoma

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## Summary

E-cadherin downregulation is related to metastatic behaviour and a poor prognosis in cancer. It might be induced by transcriptional repression mediated by the transcription factors SNAIL, ZEB1, ZEB2 and TWIST. Here, we investigated E-cadherin expression and its relationship to those transcriptional repressors (i.e. SNAIL, ZEB1, ZEB2 and TWIST) in the progression from carcinoma 'in situ' to invasion to lymph node metastasis in spontaneously arising canine invasive micropapillary carcinoma (IMPC). E-cadherin expression decreased from carcinoma in situ to invasive progression and was likely to increase with lymph node metastasis. Expression of SNAIL decreased from carcinoma in situ to invasive areas and from invasive areas to lymph nodes. Metastatic lymph nodes had higher expression of ZEB1 than carcinoma in situ and invasive areas. ZEB2 expression was observed in 52%, 38% and 33% of carcinoma in situ areas, invasive areas and lymph node metastases, respectively. TWIST expression was observed in 52%, 38% and 33% of carcinoma in situ areas, invasive areas and lymph node metastases, respectively. In invasive areas, E-cadherin downregulation correlated significantly with SNAIL and TWIST upregulation. Additionally, in infiltrating components of IMPCs, E-cadherin$^{-}$SNAIL$^{+}$ neoplastic epithelial cells were observed by immunofluorescence. Taken together, canine mammary IMPCs had a loss of E-cadherin from carcinoma in situ to invasive areas, which appears to be induced by the transcription factor SNAIL. In lymph node metastasis, ZEB1 appears to not exert E-cadherin transcriptional repression activity.



*Keywords:* dog; micropapillary carcinoma; SNAIL; transcription factors

## Introduction

E-cadherin is a calcium-dependent transmembrane protein expressed mainly by normal epithelial cells. As an adhesion molecule, E-cadherin acts to maintain the normal morphology and integrity of epithelial cells, mediating intercellular adhesion and formation and maintenance of intercellular junctions (Canel *et al.*, 2013). In cancer, E-cadherin expression by neoplastic cells may be lost, leading to loss of intercellular connections, increase in cellular motility and, consequently, enhancement of invasive behaviour, contributing to poor prognosis (Takeichi, 1993; Bracke *et al.*, 1996; Cavallaro and Christofori,

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2004). In human breast cancer, E-cadherin downregulation has been linked to poor clinicopathological characteristics (Heimann *et al.*, 2000; Rakha *et al.*, 2005), decreased survival (Guriec *et al.*, 1996; Siitonen *et al.*, 1996; Heimann *et al.*, 2000; Rakha *et al.*, 2005; Park *et al.*, 2007; Liu *et al.*, 2013) and lymph node and/or distant metastasis (Oka *et al.*, 1993; Hunt *et al.*, 1997; Rakha *et al.*, 2005).

Mechanistically, E-cadherin downregulation has been postulated to be induced by epigenetic changes that include the methylation of promoter sequences of *CDH1* (the gene responsible for E-cadherin expression), histone methylation, acetylation and ubiquitination, microRNA action (RNA molecules that modulate post-transcriptional silencing of target-genes) and transcriptional repression (Lombaerts *et al.*, 2006; Peng *et al.*, 2014). The transcriptional repression of E-cadherin is mediated mainly by zinc finger transcription factors related to the snail family (SNAIL1), zinc finger E-box binding homeobox family (zinc finger E-box binding homeobox-1 [ZEB1/δEF-1/TCF-8/AREB6] and zinc finger E-box binding homeobox-2 [ZEB2/SIP1/ZFXH1B]) and basic helix-loop-helix family (TWIST), which target specific E-boxes located in a proximal region of the *CDH1* promoter sequence (Peinado *et al.*, 2007; Drasin *et al.*, 2011; Peng *et al.*, 2014).

Spontaneously arising canine mammary carcinomas demonstrate morphological and behavioural characteristics similar to their human counterparts (Vail and MacEwen, 2000; Cassali *et al.*, 2007). Invasive micropapillary carcinoma (IMPC) in canine mammary glands (Gamba *et al.*, 2013, 2014) and its human counterpart (Luna-Moré *et al.*, 1994) are tumours with high rates of stromal and vascular invasion that are frequently associated with lymph node metastasis and a poor prognosis.

E-cadherin downregulation is closely related to the infiltrative and metastatic behaviour of cancer (Hugo *et al.*, 2007); nevertheless, the mechanisms responsible for its occurrence have not been fully elucidated. Spontaneously arising infiltrative and metastatic tumours, such as canine mammary IMPCs, could be used to determine the relationship between E-cadherin downregulation and cancer progression, helping to solve important questions concerning transcriptional repression of E-cadherin. Hence, the aim of this study was to evaluate E-cadherin expression and its relationship to *CDH1* transcriptional repressors in the progression from carcinoma in situ, to invasion, to lymph node metastasis in canine mammary IMPCs.

## Materials and Methods

### *Case Selection, Clinical Evaluation and Histopathological Analysis*

Cases of IMPC of the canine mammary gland, diagnosed from 1998 to 2014, were selected from the archives of the Veterinary School and the Laboratory of Comparative Pathology at the Institute of Biological Sciences of the Federal University of Minas Gerais and the Histopathology Laboratory of the Federal University of Bahia.

Metastases to regional lymph nodes (i.e. inguinal and axillary lymph nodes) were identified clinically and diagnosed through histopathological analysis. Metastases to non-regional lymph nodes were considered distant metastases. The distant metastasis results were based on image analysis (i.e. ultrasonography and/or radiography), necropsy examination and histopathological analysis. The IMPCs were staged according to the tumour-node-metastasis (TNM) clinical staging system for canine mammary tumours (Owen, 1980).

Tumour and lymph node specimens were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (4 μm) were stained with haematoxylin and eosin (HE). All IMPCs were reviewed and independently re-classified by two veterinary pathologists (COG and GDC) based on the histopathological characteristics described by Misdorp *et al.* (1999) and Cassali *et al.* (2014). In brief, carcinomas with cystic formations containing nests of epithelial cells with a moruliform appearance (i.e. an infiltrating micropapillary pattern), associated with or not associated with in-situ micropapillary areas, were diagnosed as IMPCs (Fig. 1A). Pure (carcinomas with a ≥75% infiltrating micropapillary pattern) and 'mixed' (carcinomas with a <75% infiltrating micropapillary pattern, associated with other infiltrating carcinomas) subtypes of IMPC were included in this study (Gamba *et al.*, 2013). The invasive areas of IMPC were graded according to the Nottingham grading system (Elston and Ellis, 1991, 1998).

### *Immunohistochemistry and Scoring*

Sections (4 μm) of primary tumours and lymph nodes were mounted on silanized slides and a peroxidase-based detection system, Advance HRP™, was applied (Dako, Carpinteria, California, USA). The slides were dewaxed in xylene and endogenous peroxidase activity was blocked with $H_2O_2$ 3% in methanol. The reagents were applied manually and immunoreactivity was 'visualised' by incubating the slides with

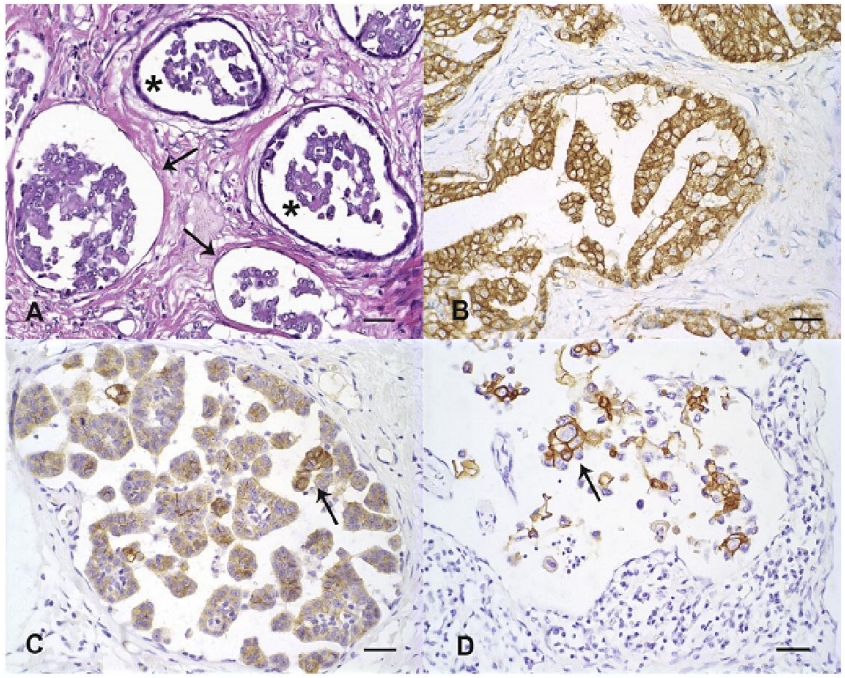


Fig. 1. Photomicrographs illustrating histopathological findings and E-cadherin immunolabelling of micropapillary carcinomas of the canine mammary gland. (A) Invasive micropapillary areas characterized by neoplastic epithelial cells within cystic formations (arrows) associated with carcinoma in situ areas (asterisks). Note that, different from invasive areas, carcinoma in situ areas are circumscribed by a basement membrane. HE. Bar, 60 µm. (B) Carcinoma in situ micropapillary area exhibiting diffuse, high-intensity membrane expression of E-cadherin. IHC. Bar, 30 µm. (C) Invasive area with multifocal, high-intensity membrane expression of E-cadherin (arrow). IHC. Bar, 30 µm. (D) Lymph node metastasis containing multifocal, high-intensity expression of E-cadherin (arrow). IHC. Bar, 30 µm.

3,3′-diaminobenzidine (Lab Vision DAB substrate system; Lab Vision, Fremont, California, USA) for 10 min. The details of antibodies, dilutions, antigen retrieval procedures and incubation times are given in Table 1. For E-cadherin, normal mammary gland was used as an internal positive control. Sections from SNAIL-, TWIST-, ZEB2- and ZEB1-positive canine tissues were used as external positive controls. Negative controls were performed using normal serum (Lab Vision Ultra V Block) in place of the primary antibody.

Analysis was performed of carcinoma in situ areas (non-invasive), invasive areas and lymph node metastasis of IMPCs. E-cadherin expression was classified based on the percentage of epithelial cells showing immunoreactivity of the cell membrane. Immunolabelled slides were scored as: negative, having no detectable labelling; +1, detectable labelling in ≤10% of the neoplastic cells; +2, detectable labelling in 10–50% of the neoplastic cells; or +3, detectable labelling in >50% of the tumour cells. IMPCs were then classified as negative (negative or 1+) or

**Table 1**
**Details of immunohistochemical methodology**

| *Target antigen* | *Clone* | *Manufacturer* | *Dilution* | *AR method* | *Incubation time (h)* |
|---|---|---|---|---|---|
| E-cadherin | 4A2C7 | Invitrogen | 1 in 80 | Water bath (98°C) | 16 |
| SNAIL | Polyclonal | Abcam | 1 in 200 | Water bath (98°C) | 1 |
| TWIST | Polyclonal | Abcam | 1 in 50 | Water bath (98°C) | 16 |
| ZEB2 | Polyclonal | Sigma–Aldrich | 1 in 100 | Water bath (98°C) | 3 |
| ZEB1 | Polyclonal | Sigma–Aldrich | 1 in 200 | Water bath (98°C) | 1 |

AR, antigen retrieval; SNAIL, zinc finger factor Snail; ZEB2, zinc finger E-box binding homeobox-2; ZEB1, zinc finger E-box binding homeobox-1.

positive (2+ or 3+) (Prasad *et al.*, 2009). To evaluate transcription factor expression, only nuclear labelling was considered. For ZEB1 and ZEB2, positivity was assessed based on the presence (+) or absence (−) of labelling (Geradts *et al.*, 2011; Karihtala *et al.*, 2013). For SNAIL and TWIST, the immunohistochemistry (IHC) results were scored as: negative, <5% of cells positive; +1, a total of 5−50% of tumour cells positive; or +2, a total of >50% of tumour cells positive (Waldmann *et al.*, 2009). IMPCs were classified as either 0−+1 (negative) or +2 (positive).

*Confocal Fluorescence Microscopy*

In order to elucidate the relationship between the transcription factor SNAIL and E-cadherin, triple labelling immunofluorescence was performed in four cases of IMPC that were immunohistochemically positive for SNAIL in invasive areas. Confocal immunofluorescence was performed as previously described with minor modifications (Rodrigues *et al.*, 2007, 2008; Gomes *et al.*, 2008). In brief, formalin-fixed and paraffin wax-embedded tissue sections were dewaxed, rehydrated and unmasked in 'trilogy solution' (Cell Marque, Koclin, California, USA) under pressurized heating (125°C) for 20 min according to the manufacturer's instructions. Next, samples were rinsed in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer solution, pH 7.4) (Sigma−Aldrich, Carlsbad, California, USA) and then they were incubated in PBS containing 0.2% Triton X-100 (Sigma−Aldrich) for 20 min and blocked in PBS containing 1% bovine serum albumin (BSA, Sigma−Aldrich) for 30 min. The sections were next incubated with a mouse monoclonal antibody against E-cadherin (1 in 80 dilution, clone 4A2C7, Invitrogen, Carlsbad, California, USA) and a rabbit polyclonal antibody against SNAIL (1 in 200 dilution, polyclonal, Abcam, Cambridge, UK) overnight at 4°C and then they were rinsed three times for 5 min in PBS. Subsequently, sections were incubated with Alexa Fluor® 488 goat anti-mouse IgG antibody (1 in 1,000 dilution, Life Technologies, Karlsruhe, Germany), Alexa Fluor® 555 goat anti-rabbit IgG antibody (1 in 1,000 dilution, Life Technologies) and Hoechst 33,258 (1 μg/ml, Life Technologies) for 1 h at room temperature. Next, samples were washed three times in PBS for 10 min and then mounted with Prolong Gold Antifade™ reagent (Life Technologies). Primary antibodies were omitted as a negative control. Images were collected using a Zeiss LSM 5 Live (Carl Zeiss, Jena, Germany) confocal microscope using an oil ×40 1.3 NA objective lens. Samples were excited at 405 nm and observed at 415−480 nm to detect Hoechst; excited at 488 nm and observed at 500−525 nm to detect Alexa Fluor 488; and excited at 532 nm and observed using a LP550 filter to detect Alexa Fluor 555. For SNAIL and E-cadherin, nuclear and cell membrane labelling was considered, respectively. Neoplastic epithelial cells were classified in four combinatorial phenotypic groups: E-cadherin$^+$/SNAIL$^+$, E-cadherin$^+$/SNAIL$^-$, E-cadherin$^-$/SNAIL$^+$ and E-cadherin$^-$/SNAIL$^-$. The number of neoplastic epithelial cells in each group was counted in 10 invasive areas of IMPC.

*Statistical Analysis*

The GraphPad Prism 5.0 software package was used for the statistical analysis. A comparison between the protein expression in the carcinoma in situ, invasive and lymph node areas of IMPC was performed using a two-sided Fisher's exact test. Spearman's correlation test was used to estimate the relationship between the labelling patterns of different antibodies used in the carcinoma in situ, invasive and lymph node areas. $P < 0.05$ was considered significant for all statistical tests.

## Results

*Epidemiological and Clinicopathological Data*

In total, 34 IMPCs of the canine mammary gland were retrieved. Some epidemiological and clinicopathological data were not available for all cases (Table 2). The age of the animals ranged from 7 to 15 years (mean 11.20 ± 2) at the time of surgery and most were purebred bitches (21/33, 64%). Multicentric localization (15/32, 47%) and tumours >5 cm in size were predominant (14/31, 45%). Regional and distant metastases were observed in 100% (27/27) and 33% (7/21) of the dogs, respectively, and stage IV was the most common clinical stage (11/16, 69%). Histological grade II (23/34, 68%) and the pure subtype (20/34, 59%) were more frequently observed microscopically. In-situ (non-invasive) ductal carcinomas associated with invasive lesions were observed in 85% (29/34) of IMPCs.

*Immunohistochemistry*

Immunohistochemical analysis was performed in all primary IMPCs and in 21/27 (78%) lymph node metastases. In six samples of lymph nodes, IHC was not possible due to the loss of immunoreactivity. The proportion of E-cadherin-positive results decreased from

carcinoma in situ to invasive progression (85% and 42%, respectively; $P = 0.001$) (Figs. 1B and C) and was likely to increase with lymph node metastasis when compared with invasive components (42% in invasive areas versus 52% in lymph nodes) (Fig. 1D). A greater percentage of ZEB1-positive samples was observed in lymph nodes (57%) than in the carcinoma in situ and invasive areas (21% and 18%, respectively) (carcinoma in situ × lymph node, $P = 0.01$; invasive × lymph node, $P = 0.006$) (Figs. 2A and B). Concerning ZEB2, positive immunolabelling was observed in 52%, 38% and 33% of carcinoma in situ areas, invasive areas and lymph node metastases, respectively. A decrease in SNAIL-positive expression was revealed from carcinoma in situ to invasive areas (72% and 44%, respectively; $P = 0.04$) (Figs. 2C and D) and from invasive areas to lymph nodes (44% and 14%, respectively; $P = 0.03$). Regarding TWIST, 41%, 28% and 0% of carcinoma in situ areas, invasive areas and lymph node metastases, respectively, were classified as positive. Table 3 summarises the frequency of canine mammary IMPCs and lymph node metastases positive for the different markers.

For carcinoma in situ areas and lymph node metastases, a weak and non-significant correlation between the markers was observed. For invasive areas, E-cadherin downregulation had a significant correlation with both SNAIL ($r = -0.377$, $P < 0.01$) and TWIST ($r = -0.310$; $P = 0.05$) upregulation.

**Table 2**
**Clinicopathological features of IMPC in canine mammary glands**

| *Characteristics* | *n=* | *IMPC, n (%)** |
|---|---|---|
| Breed | 33 | |
| Pure-breed | | 24 (73) |
| Cross-breed | | 9 (27) |
| Tumour size | 31 | |
| <3 cm | | 5 (16) |
| 3–5 cm | | 12 (39) |
| >5 cm | | 14 (45) |
| Lymph node metastasis† | 27 | |
| N0 | | 0 (0) |
| N1 | | 27 (100) |
| Distant metastasis‡ | 21 | |
| M0 | | 14 (67) |
| M1 | | 7 (33) |
| Stage (TNM)§ | 16 | |
| I | | 0 (0) |
| II | | 0 (0) |
| III | | 0 (0) |
| IV | | 11 (69) |
| V | | 5 (31) |
| Carcinoma in situ areas | 34 | |
| No | | 5 (15) |
| Yes | | 29 (85) |
| Subtype of IMPC‖ | 34 | |
| 'Pure' | | 20 (59) |
| 'Mixed' | | 14 (41) |
| Histological grade¶ | 34 | |
| I | | 8 (23) |
| II | | 23 (68) |
| III | | 3 (9) |

*Some data were not available for all 34 IMPCs.
†N0, absence of regional metastasis; N1, regional metastasis.
‡M0, absence of distant metastasis; M1, distant metastasis.
§I (T1N0M0), II (T2N0M0), III (T3N0M0), IV (T1, 2, 3N1M0) and V (T1, 2, 3N0, 1M1) (Owen, 1980).
‖'Pure' subtype: carcinomas with a ≥75% infiltrating micropapillary pattern; 'mixed' subtype: carcinomas with a <75% infiltrating micropapillary pattern, associated with other infiltrating carcinomas (Gamba *et al.*, 2013).
¶Histological grading based on that of Elston and Ellis (1991, 1998).

*Immunofluorescence*

Most of the IMPC invasive areas had a predominance of E-cadherin$^{+}$SNAIL$^{+}$ (case 1, 68.2%; case 4, 67.4%) and E-cadherin$^{-}$SNAIL$^{+}$ (case 2, 38.9%; case 3, 49.9%) neoplastic epithelial cells (Table 4, Fig. 3).

## Discussion

E-cadherin downregulation has frequently been related to the infiltrative and metastatic behaviours of neoplasms (Cavallaro and Christofori, 2004). Its occurrence has been widely studied through in-vitro cell culture and in-vivo animal models (Wendt *et al.*, 2011; Vergara *et al.*, 2015); however, to gain insight into this topic, spontaneously arising metastatic tumours should be studied. The present study investigates spontaneously arising canine mammary IMPCs that usually have infiltrative and metastatic behaviour (Gamba *et al.*, 2013, 2014). This tumour has a high clinical stage, high rates of lymph node metastasis and high rates of carcinoma in situ areas associated with invasive components, so provides an excellent opportunity to investigate E-cadherin downregulation in cancer progression.

Canine IMPCs showed E-cadherin-loss in carcinoma in situ areas compared with invasive areas, as has been described in human breast cancer (Knudsen *et al.*, 2012; Choi *et al.*, 2013). E-cadherin downregulation has also been described in invasive areas of some special types of human breast cancer, such as IMPCs and medullary carcinomas (Nagi *et al.*, 2005; Aleskandarany *et al.*, 2014). These findings suggest that in malignant neoplastic lesions, E-cadherin downregulation could be a critical tool, adopted for neoplastic cells, to move beyond the basement membrane and infiltrate into the surrounding stroma.

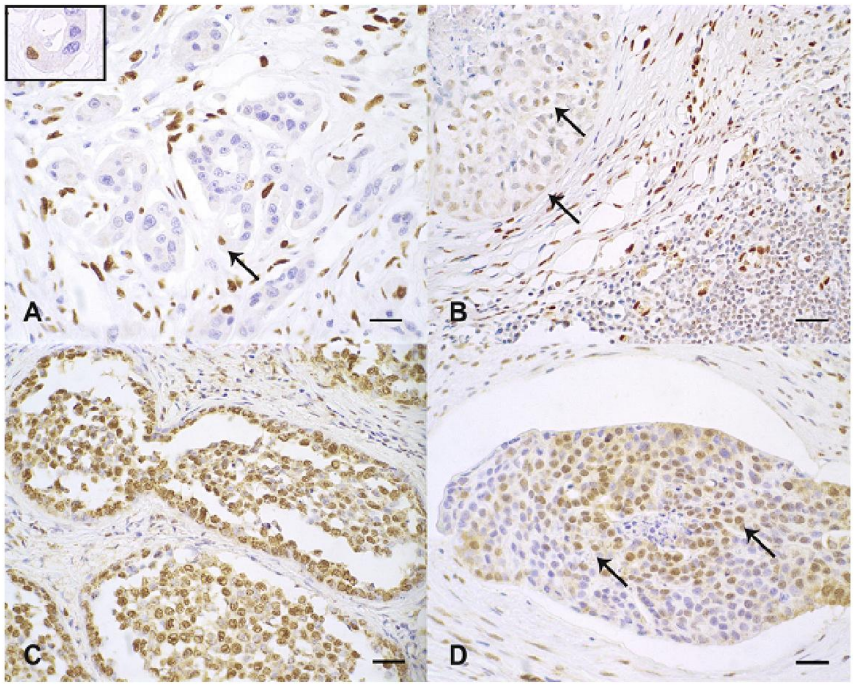


Fig. 2. Photomicrographs illustrating ZEB1 and SNAIL expression in micropapillary carcinomas of the canine mammary gland. (A) Focal nuclear expression of ZEB1 in invasive micropapillary areas (arrow) (inset shows detail). IHC. Bar, 30 µm. (B) Lymph node metastasis with multifocal nuclear expression of ZEB1 (arrows). IHC. Bar, 30 µm. (C) Carcinoma in situ micropapillary areas exhibiting diffuse nuclear expression of SNAIL. IHC. Bar, 30 µm. (D) Invasive micropapillary area with multifocal nuclear expression of SNAIL (arrows). IHC. Bar, 30 µm.

**Table 3**
**Frequency of canine mammary IMPCs and lymph node metastases positive for E-cadherin, ZEB1, ZEB2, SNAIL and TWIST**

| *Antigen* | *Positive IMPCs (IHC)/Total (%)* | | |
|---|---|---|---|
| | *Carcinoma* in situ | *Invasive* | *Lymph node* |
| E-cadherin | 22/26 (85)[a] | 13/31 (42)[b] | 11/21 (100)[b] |
| ZEB1 | 6/28 (21)[a] | 6/33 (18)[a] | 12/21 (57)[b] |
| ZEB2 | 15/29 (52)[a] | 13/34 (38)[a] | 7/21 (33)[a] |
| SNAIL | 2129 (72)[a] | 15/34 (44)[b] | 3/21 (14)[c] |
| TWIST | 11/27 (41)[a] | 9/32 (28)[a] | 0/16 (0)[b] |

IMPC, invasive micropapillary carcinoma; IHC, immunohistochemistry; ZEB1, zinc finger E-box binding homeobox-1; ZEB2, zinc finger E-box binding homeobox-2; SNAIL, zinc finger factor SNAIL. Statistical analysis was performed using Fisher's exact test and in each row different letters denote statistical significance at $P < 0.05$.

One of the mechanisms in E-cadherin downregulation involves epigenetic events such as transcriptional repression mediated by transcription factors (Thiery, 2002). Strong evidence that the transcriptional repressor SNAIL is related to E-cadherin downregulation has been provided through studies of murine and/or human cell lines (Cano *et al.*, 2000; Yang *et al.*, 2004, 2012). In man, this hypothesis has been suggested following examination of surgical specimens of breast cancer (Van Nes *et al.*, 2012; Cao *et al.*, 2015), lung adenocarcinomas (Maeng *et al.*, 2014), cutaneous and cervical squamous cell carcinomas (Lee *et al.*, 2008; Chen *et al.*, 2013; Zhao *et al.*, 2013), parathyroid carcinomas (Fendrich *et al.*, 2009), malignant pleural mesotheliomas (Kobayashi *et al.*, 2013), gastric cancer (Rosivatz *et al.*, 2002), uterine endometrial cancer (Tanaka *et al.*, 2013) and hepatocellular carcinomas (Yang *et al.*, 2009). In spontaneously arising canine mammary IMPCs, we observed higher expression of SNAIL in carcinoma in situ areas compared with invasive areas. In addition, in invasive areas, this molecule had an inverse correlation with E-cadherin and, at times, through immunofluorescence, was observed to be associated with E-cadherin-negative areas. Therefore, in canine IMPCs, SNAIL might be one of the important regulators of E-cadherin expression and its transcriptional repression seems to start in carcinoma in situ areas and have its effect in invasive areas.

In the present study, trends towards an increase in E-cadherin expression in invasive areas compared with lymph node metastases were observed. This has also been described in nodal and non-nodal distant metastasis in human breast neoplasms (Bukholm *et al.*, 2000; Kowalski *et al.*, 2003;

**Table 4**
**The number of neoplastic epithelial cells expressing E-cadherin (ECAD) and SNAIL in invasive areas of IMPC of the canine mammary gland**

| *Case* | *Number of epithelial neoplastic cells counted (%)** | | | | |
|---|---|---|---|---|---|
| | *Total* | *ECAD⁻SNAIL⁻* | *ECAD⁻SNAIL⁺* | *ECAD⁺SNAIL⁻* | *ECAD⁺SNAIL⁺* |
| Case 1 | 421 (100) | 11 (2.6) | 97 (23) | 26 (6.2) | 287 (68.2) |
| Case 2 | 352 (100) | 73 (20.7) | 137 (38.9) | 22 (6.3) | 120 (34.1) |
| Case 3 | 491 (100) | 62 (12.6) | 245 (49.9) | 16 (3.3) | 168 (34.2) |
| Case 4 | 420 (100) | 13 (3.1) | 6 (1.4) | 118 (28.1) | 283 (67.4) |

*Total number of epithelial neoplastic cells counted in 10 fields of invasive areas of IMPC of the canine mammary glands.

Chao *et al.*, 2012). It is consistent with a mesenchymal–epithelial transition that enables neoplastic cells to form a metastatic focus by recovering E-cadherin expression and intercellular adhesion (Kowalski *et al.*, 2003). Additionally, in lymph node metastases we observed an increased number of ZEB1-positive cells, which could indicate an absence of *CDH1* transcriptional repression of this molecule in cancer metastasis. It may be related to a failure of ZEB1 post-translational

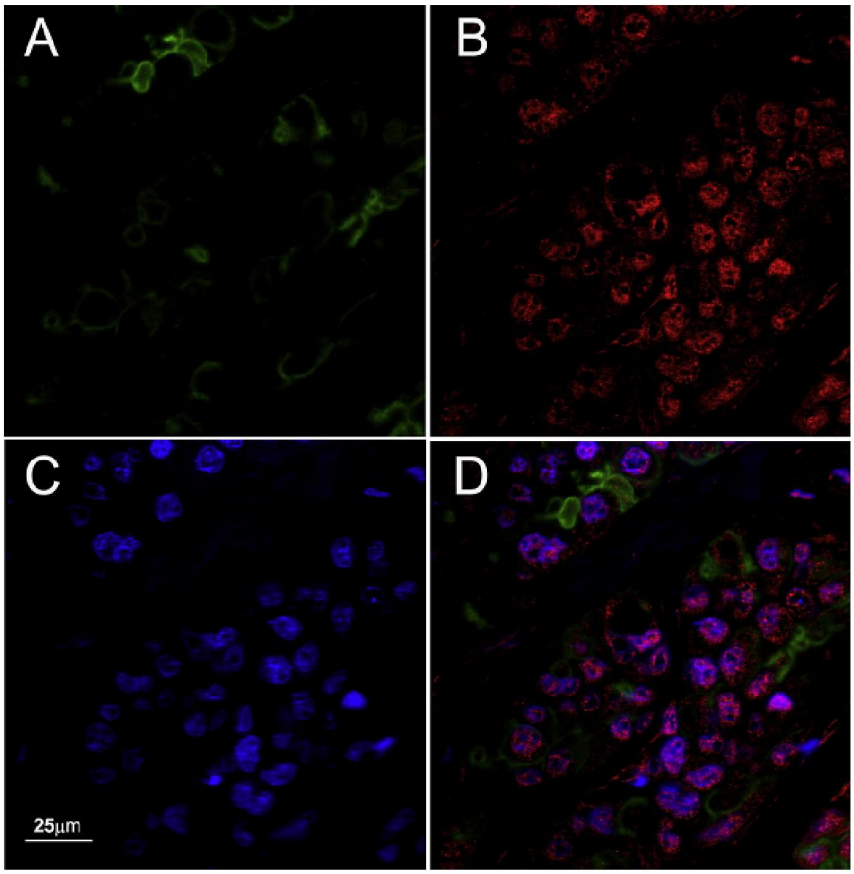


Fig. 3. Confocal immunofluorescence images showing the nuclear and cytoplasmic membrane expression of SNAIL and E-cadherin, respectively, in invasive areas of IMPC of the canine mammary gland. (A) Confocal immunofluorescence image of cytoplasmic membrane E-cadherin (green). (B) Nuclear SNAIL (red). (C) Nuclear staining with Hoechst (blue). Note that most of the neoplastic cells are positive for SNAIL and negative for E-cadherin. (D) Nuclear localization of SNAIL was confirmed by the merged image, which demonstrates the co-localization of SNAIL with Hoechst. Images are representative of 10 fields from four separate primary tumour cases. Bar, 25 µm.

phosphorylation (mandatory to ZEB1 activity) and/or to the overwhelming of ZEB1 silencing by other transcription factors that positively regulate E-cadherin transcription (Putzke *et al.*, 2011).

Taken together, canine mammary IMPCs allow investigation of the mechanisms of E-cadherin down-regulation in cancer progression. There is loss of E-cadherin expression in carcinoma in situ areas compared with invasive areas and this seems to be induced by the transcription factor SNAIL. In lymph node metastases, ZEB1 appears not to exert E-cadherin transcriptional repression activity.

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## Conflict of Interest Statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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## 6. CONCLUSÕES FINAIS

A partir das condições e dos dados obtidos nesta pesquisa pode-se concluir que:

- Os CMI apresentam perda da expressão de E-caderina na progressão de áreas carcinomatosas *in situ* para áreas invasoras o que é induzido pela repressão transcricional de SNAIL;

- Em metástases para linfonodo de CMI, a proteína ZEB1 não está relacionada à repressão transcricional de E-caderina;

- ZEB2 citoplasmático é um fator importante em estágios precoces da progressão maligna e sua expressão está relacionada com sobrevida global curta de cadelas portadoras de CMI mamários;

- A ausência da expressão de ZEB1 está associada com o processo de perda de diferenciação de CMI mamários caninos.

## 7. CONSIDERAÇÕES FINAIS

Assim como foi relatado na presente tese, na execução do estudo de mestrado (GAMBA et al., 2013) e nos dados apresentados no artigo em **APÊNDICE D**, CMI mamários na espécie canina, são neoplasias que apresentam inúmeras similaridades morfológicas e de comportamento biológico com CMI humanos, dentre estas podendo-se destacar sobrevida global curta e altas taxas de metástase para linfonodos regionais. Diante destes resultados pode-se inferir que CMI em cadelas poderiam ser considerados excelentes modelos espontâneos para a elucidação dos mecanismos relacionados com potencial invasivo e metastatizante dos CMI humanos. Neste estudo, conseguimos demonstrar que, em caninos, os CMI apresentam eventos relacionados com a TEM, como a perda da expressão de E-caderina provavelmente induzida pelo repressor transcricional SNAIL; além disso, relatamos que moléculas participantes da TEM, como o ZEB2, podem estar relacionadas com sobrevida global curta de cadelas portadoras de CMI.

No **APÊNDICE D** foram relatadas inúmeras similaridades clinicopatológicas, imunofenotípicas e evolutivas entre CMI nas espécies humanas e canina, porém investigações avaliando marcadores da TEM nas amostras humanas poderiam ser futuramente realizados, para com isso pesquisarmos o papel destas moléculas em mulheres portadoras de CMI mamários.

Além do que foi obtido neste estudo, no período de doutoramento, outras avaliações foram realizadas e darão origem ao terceiro e quarto artigos:

O terceiro artigo tem por objetivo avaliar a transcrição de RNAm e a expressão proteica de E-caderina e seu repressor transcricional ZEB2 em amostras de CMI caninos imunohistoquimicamente positivas e negativas para ZEB2. Para avaliação da expressão proteica para ZEB2 realizou-se imuno-histoquímica dos 32 casos de CMI caninos obtidos. A avaliação da imunoexpressão para ZEB2 foi realizada sendo considerados positivos casos com qualquer positividade nuclear para o marcador, e negativos casos com ausência de marcação nuclear. Posteriormente, selecionou-se 10 CMI positivos e 10 CMI negativos, nos quais realizou-se as técnicas de imunofluorescência, com dupla marcação para E-caderina e ZEB2; e a técnica de hibridização *in situ* de RNAm (kit QuantiGene ViewRNA ISH Tissue

Assay (Affimetrix) com sondas customizadas para os genes responsáveis pela expressão das proteínas ZEB2 e E-caderina (gene CDH1) em caninos. Os experimentos relacionados a este estudo já foram realizados restando apenas a avaliação das amostras de imunofluorescência e de hibridização *in situ* e posterior análise estatística dos dados.

O quarto artigo tem por objetivo avaliar a expressão proteica e de RNAm de vimentina em CMI da glândula mamária canina e correlacioná-las com fatores clinicopatológicos e sobrevida global de cadelas portadoras desta neoplasia. Para avaliação da expressão proteica realizou-se imuno-histoquímica para vimentina dos 32 casos de CMI caninos obtidos. Destes, selecionou-se 20 CMI, nos quais realizou-se a técnica de hibridização *in situ* de RNAm com utilização de sondas customizadas para o gene responsável pela expressão da proteína vimentina em cães. Os experimentos relacionados a este estudo já foram realizados restando apenas a avaliação das amostras hibridização *in situ* e posterior análise estatística dos dados.

A técnica de imunofluorescência para o terceiro artigo, acima descrito, foi executada no laboratório de imunologia celular e molecular/Instituto de Ciência Biológicas/UFMG com a colaboração do Prof. Dr. Dawidson Assis Gomes e da Dra. Michele Rodrigues; a técnica de hibridização *in situ* foi realizada no Laboratório de Patologia do Centro Internacional de Pesquisa (CIP) do Hospital do Câncer A.C. Camargo com a colaboração do Prof. Dr. Rafael Malagoli Rocha e da doutoranda Mariana Rezende Alves.

Estes trabalhos contribuirão para um melhor conhecimento da repressão transcricional, responsável pela ocorrência da TEM em CMI, e do papel de moléculas relacionada à TEM, como a vimentina, no prognóstico de CMI mamários caninos.

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# 9. APÊNDICES

## APÊNDICE A - ATIVIDADES DESENVOLVIDAS NO PERÍODO DO DOUTORADO

### EXPERIÊNCIA PROFISSIONAL

- Participação no projeto de extensão "Diagnóstico citológico e histopatológico de biópsias caninas" do Laboratório de Patologia Comparada da Universidade Federal de Minas Gerais (UFMG): 11/2009 – atual.

- Professor Voluntário da Disciplina de Patologia Geral na UFMG: 03/2012-07/2012.

- Professor substituto das disciplinas de Histologia Veterinária I e II, Patologia Geral e Especial, Doenças das Aves e Doenças dos Suínos na Universidade Federal de Santa Catarina (UFSC): 07/2015-12/2015.

## APÊNDICE B - PRODUÇÃO CIENTÍFICA RELACIONADA À DISSERTAÇÃO NO PERÍODO DE MARÇO DE 2012 A JANEIRO DE 2016

### RESUMOS SIMPLES ENVIADOS PARA APRESENTAÇÃO EM EVENTOS

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## APÊNDICE C - TRABALHOS NÃO RELACIONADOS À TESE

## RESUMOS ENVIADOS PARA APRESENTAÇÃO EM EVENTOS

### RESUMOS SIMPLES

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**APÊNDICE D - RESULTADOS COMPLEMENTARES À TESE APRESENTADOS NO FORMATO DE UM ARTIGO CIENTÍFICO INTITULADO "INVASIVE MICROPAPILLARY CARCINOMA OF THE MAMMARY GLAND IN HUMAN AND CANINE SPECIES: CLINICOPATHOLOGICAL, IMMUNOPHENOTYPICAL AND SURVIVAL APPROACH".**

# INVASIVE MICROPAPILLARY CARCINOMA OF THE MAMMARY GLAND IN HUMAN AND CANINE SPECIES: CLINICOPATHOLOGICAL, IMMUNOPHENOTYPICAL AND SURVIVAL APPROACH

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SUMMARY

Invasive micropapillary carcinoma (IMPC) of the human breast is a neoplasm that show proclivity to lymph node metastasis and lymphatic invasion. In canine species it has just been reported in mammary gland and appears to show histological and biological aspects similar to its human counterpart. Thus, the aim of this study was to evaluate, comparatively, clinicopathological, immunohistochemical and prognostic characteristics of the mammary IMPC in canine and human species. Canine neoplasms showed higher medium size than human tumours. Moreover, in canine species regional and distant metastasis were more frequently observed. The histopathological appearance was similar in canine and humans IMPCs, both revealing predominance of moderate histological grade. However, the pure subtype and neoplastic emboli were more frequently observed in canines. Concerning immunohistochemical evaluation, most of canine and human IMPCs were estrogen and progesterone receptor-positive; and HER-2-negative. The reversed pattern of EMA and high proliferation index predominated in both species. During the follow up period, the mortality due to the neoplastic disease was more frequently observed in canines (94%) than in humans (4%). Hence, human and canine IMPCs demonstrated to be aggressive neoplasms with high rates of lymph node metastasis and high proliferation index. Canine IMPCs seems to be more aggressive neoplasms with larger tumour size, and higher rates of pure subtype, regional and distant metastasis and mortality, when compared to its human counterpart.

**Keywords:** breast cancer; lymph node metastasis; immuhistochemistry; comparative oncology.

INTRODUCTION

Invasive micropapillary carcinoma (IMPC) of the breast in humans is a rare variant of ductal carcinoma firstly described for Siriaunkgul and Tavassoli (1993) and recognized as an independent histological subtype of carcinoma for the World Health Organization (WHO) in 2003 (TAVASSOLI; DEVILEE, 2003). This neoplasm is considered a lymphotropic and metastasising phenotype of the breast cancer, because it is usually associated with regional and distant metastasis, and lymphatic invasion (LUNA-MORÉ et al., 2000; MIDDLETON et al., 1999).

Histopathologically IMPCs show multiple cystic formations filled out with nests of epithelial cells lacking fibrovascular cores (DE LA CRUZ et al., 2004; SIRIAUNKGUL; TAVASSOLI, 1993). These nests usually show immunostaining for epithelial membrane antigen (EMA), which is applied in order to determinate the definitive diagnosis of the neoplasm (NASSAR et al., 2004). In addition, IMPC may be associated with other morphological patterns of carcinomas, thereafter being considered mixed IMPCs (MARCHIÒ et al., 2009). Regarding the prognostic markers, IMPCs usually reveal overexpression for human epidermal growth factor receptor-2 (HER-2), high rates of estrogen receptor (ER) and progesterone receptor (PR) positivity and high cellular proliferation index (LUNA-MORE et al., 1996; MARCHIO et al., 2008; MIDDLETON et al., 1999; PATERAKOS et al., 1999; TRESSERRA et al., 1998; WALSH; BLEIWEISS, 2001; YAMAGUCHI et al., 2010).

In animals, micropapillary carcinomas have been described in canine, feline and equine species (GAMBA et al., 2011, 2013; SEIXAS et al., 2007). In bitches this neoplasm was firstly reported for Cassali et al. (2002, 1999), revealing histological and biological aspects similar to human IMPCs. In canine species, IMPCs showed positivity for EMA, hormonal receptors (ER and PR), cytokeratin (CK) 34βE12 and nuclear EGFR. In addition revealed negativity for HER-2 and cellular proliferation index of 14.8% (GAMBA et al., 2013; RODRIGUES et al., 2015).

In spite of the biological, clinicopathological and immunophenotipical similarities between human and canine IMPCs, comparative studies, evaluating concomitantly both species, have not been published yet. These investigations could contribute to the consolidation of canine

species as a spontaneous model of human breast IMPC. Hence, the aim of this study was to evaluate, comparatively, clinicopathological, immunohistochemical and prognostic characteristics of the mammary IMPC in canine and human species.

## MATERIALS AND METHODS

### CASE SELECTION, CLINICAL EVALUATION AND HISTOPATHOLOGICAL ANALYSIS

Cases of IMPC of the canine mammary gland were selected from the archives of the Veterinary School and the Laboratory of Comparative Pathology at the Institute of Biological Sciences of the Federal University of Minas Gerais and the Histopathology Laboratory of the Federal University of Bahia. The samples of human breast IMPCs were obtained from A.C. Camargo Cancer Center, São Paulo/SP. Canines and humans showing the diagnosis of IMPC associated with clinical signs of inflammatory carcinoma were eliminated of the study.

For both species, metastases to regional lymph nodes were clinically identified and diagnosed through histopathological analysis. Metastases to non-regional lymph nodes were considered distant metastases. The distant metastasis results were based on image analysis (ultrasonography and/or X-ray), necropsy examination, and histopathological analysis.

In order to perform the histopathological analysis, the tumour specimens were fixed in 10% neutral buffered formalin and embedded in paraffin wax, and 4-µm-thick histological sections were cut and stained with haematoxylin and eosin. All canine IMPCs were reviewed and independently re-classified by two veterinary pathologists (COG and GDC) based on the histopathological characteristics described by Misdorp et al. (1999) and Cassali et al. (2014). Human specimens of IMPC were diagnosed by a human pathologist (VPA) based on WHO classification (TAVASSOLI; DEVILEE, 2003). In brief, for human and canine species, carcinomas with cystic formations containing nests of epithelial cells with a moruliform appearance (infiltrating micropapillary pattern), associated with or not with ‘in situ’ micropapillary areas, were diagnosed as IMPCs. Pure (carcinomas with a ≥75% infiltrating micropapillary pattern) and ‘mixed’ (carcinomas with a <75% infiltrating micropapillary pattern, associated with other infiltrating carcinomas) subtypes of IMPC were included in this study (GAMBA et al., 2013; MIDDLETON et al., 1999; ZEKIOGLU et al., 2004). The

invasive areas of canine and human IMPC were graded according to the Nottingham grading system (ELSTON; ELLIS, 1991, 1998).

All experimental procedures were performed with the approval of the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais and A.C. Camargo Cancer Center, São Paulo/SP.

**IMMUNOHISTOCHEMISTRY AND SCORING**

For the immunohistochemical analysis, consecutive 4-µm-thick sections of primary tumours were mounted on silanised slides, and for canine and human IMPC, a peroxidase-based detection system, Advance HRP (Dako North America Inc., Carpinteria, California, USA), and a Novolink™ Polymer Detection System (Leica Biosystems Newcastle Ltd, New Castle, UK) were applied, respectively. Endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution in methyl alcohol. The reagents were applied manually, and immunoreactivity was visualised by incubating the slides with diaminobenzidine (Lab Vision DAB substrate system) for 10 min. The details regarding antibodies, dilutions, antigen retrieval procedures, and incubation times for the immunostaining process are shown in **Table 1**. Sections from a HER-2, ER, PR, EMA and Ki-67 positive canine mammary carcinoma and human carcinoma were used as a positive control. Negative controls were assessed using normal serum in the place of the primary antibody when analysing a sample of human and canine IMPC.

HER-2 expression was determined by cell membrane staining and scored according to the guidelines established by the American Society of Clinical Oncology, College of American Pathologists (ASCO/CAP) (WOLFF et al., 2006). Staining for ER and PR was evaluated, and cases scored positive if nuclear staining was present in ≥1% of the tumour cells (HAMMOND et al., 2010). Regarding Epithelial Membrane Antigen (EMA) two staining patterns were evaluated in nests of epithelial cells: intracytoplasmic pattern and classical reversed polarity pattern (lineal staining in external membrane of tumour cell clusters). The proliferative index was calculated by counting the number of nuclei positive for Ki-67 staining in a total of 1000 neoplastic cells from each lesion (DUTRA et al., 2008). For both species the proliferation index was considered high when showed values ≥ 10% (IACOPETTA et al., 1998; SESHADRI et al., 1996).

**SURVIVAL TIME**

The overall survival time was defined as the period (in days) between surgery and death due to the tumour. The follow-up period was 1685 days for humans and 404 days for canines. The cause of death was confirmed at the postmortem examination. Animals and humans that died from unknown causes or from causes unrelated to the tumour were censored.

**STATISTICAL ANALYSIS**

The Statistica software package was used for the statistical analysis. The comparisons between the clinicopathological parameters (Age, tumour size, lymph node metastasis, distant metastasis, subtype of IMPC, histological grade, vessel invasion) and immunohistochemical findings of canine and human IMPC were performed using a nonparametric Mann-Whitney U Test. The survival rate was calculated using the Kaplan–Meier method, and statistical significance was examined using a log-rank test. Probability values below 0.05 were considered significant for all statistical tests.

RESULTS

In total were obtained 25 and 20 cases of human and canine IMPCs, respectively. The ages of the animals at the time of surgery ranged from 7 to 13 years (mean 10.95 years ± 1.80 years); for humans the age ranged from 28 to 87 years (mean 54 years ± 15 years). Concerning the tumour size, canine neoplasms showed higher size than human tumours (canines, 5.94±3.31cm; humans, 2±1.80 (9-0.50); p= 0.009). Moreover, in canine species, regional (p=0.007) and distant metastasis (p= 0.005) were more frequently observed.

Regarding histopathological evaluation, all canine and humans IMPCs demonstrated numerous irregular stromal cystic formations containing nests of epithelial cells in a “morule like” pattern (**Figure 1A and 1D**). The pure pattern predominated in canine species (p=0.01). Regarding the subtypes of IMPC, in dogs, mixed pattern IMPC components showed association with papillary carcinoma (4/6, 66.66%), carcinosarcoma (1/6, 16.66%) and carcinoma in mixed tumour (1/6, 16.66%); in humans, mixed IMPCs were associated with infiltrating ductal carcinoma not otherwise specified (15/17, 88.23%), mucinous carcinoma (1/17, 5.88%) and lobular carcinoma (1/17, 5.88%). For both species the majority of cases

were of moderate histological grade. Neoplastic emboli were more frequently observed in canine species (p=0.03).

Regarding immunohistochemical evaluation, most of canine and human IMPCs were ER and PR positive (**Figure 1B and 1E**); and HER-2-negative (canines, 15/20, 75%; humans, 20/25, 80%). The reversed pattern of EMA (**Figure 1C and 1F**) and high proliferation index predominated in both species. The **table 2** shows clinicopathological and immunohistochemical data of the canine and human species.

Survival data were available for 16 dogs, and 15 animals (94%) died as a consequence of the mammary neoplasia; one animal (16%) died due to haemorrhagic diathesis 8 days after the surgery. The median overall survival time of canine IMPC was 120 days. For human IMPC, survival data were available for 25 patients and of these one women (4%) died due to the disease 1685 days after the surgery; two patients (8%) were alive with distant metastasis of IMPC (one woman with bone metastasis and another with pleural metastasis); 18 patients (72%) were alive without neoplastic disease evidence; one patient (4%) died due to a retinoblastoma; and the remaining three patients (12%) were missed during the follow-up.

## DISCUSSION

IMPCs of the woman are an aggressive subtype of ductal carcinoma, which rarely occurs in breast, and are associated with poor overall survival and high rates of lymph node metastasis (LUNA-MORE et al., 1994). In bitches this neoplasm has just been reported in mammary gland showing pathological and behavioural similarities with its human counterpart (GAMBA et al., 2013). To our knowledge, it was the first research that compares, concomitantly, clinicopathological, immunophenotypical and survival findings of IMPCs in canine and human species.

In IMPC cases, using the age conversion table, postulated by Lebeau (1953), the average age observed for canine IMPC could be compared to a 43.8 years old woman, different from the 54 years old average age of humans IMPCs evaluated in our study. In other researches the human IMPC average has varied from 50 to 62 years old (DE LA CRUZ et al., 2004; LUNA-MORÉ et al., 2000; MIDDLETON et al., 1999; NASSAR et al., 2001; PATERAKOS et al.,

1999; PETTINATO et al., 2004; SIRIAUNKGUL; TAVASSOLI, 1993; TRESSERRA et al., 1998; WALSH; BLEIWEISS, 2001; ZEKIOGLU et al., 2004).

Surprisingly, in dogs, it was observed larger tumour size, higher frequency of regional and distant metastasis, and vascular invasion, demonstrating that, in this species, IMPC appears to be more aggressive than its human counterpart. To our experience, the predominance of larger tumour size, might be related to the late diagnosis of mammary neoplasms which is frequent in veterinary clinical routine. In canine malignant mammary tumours it has been reported that high size (>5cm) neoplasms may be associated with high proliferation index and poor overall survival (FERREIRA et al., 2009). Moreover Klein (2009) postulated that the increase of tumour size could generate clones of neoplastic cells with higher metastasising behaviour, what could be occurring in canine IMPC.

Concerning the histopathological features, both species revealed classical stromal invasion with “morule like” pattern of moderately differentiated (grade II) neoplastic cells inside cystic formations. Nevertheless, the pure subtype of IMPC predominated in bitches in relation to humans. It is similar to the literature findings related to human beings (LUNA-MORÉ et al., 2000; WALSH; BLEIWEISS, 2001) and canine species (GAMBA et al., 2015). We postulate that differences in gene expression might be influencing the distinct phenotype revealed for the dogs. Canine IMPCs, different from its human counterpart, have just been related to expression of proteins such as EGFR, ZEB2 and SNAIL which may be related to its morphological appearance (GAMBA et al., 2014, 2015; RODRIGUES et al., 2015).

Concerning the immunohistochemical markers, despite being an aggressive neoplasm, we observed that canine IMPC, as such as its human counterpart, showed positivity for hormonal receptors (ER and PR) and negativity for HER-2. It has already been reported in humans (WALSH; BLEIWEISS, 2001; YAMAGUCHI et al., 2010; ZEKIOGLU et al., 2004), and indicates the well differentiated character of this neoplasm that also appears to occur in dogs. Regarding the staining for EMA, here we observed in both species, predominance of the classical reversed pattern widely described for IMPCs (GAMBA et al., 2013; LI et al., 2006; LUNA-MORÉ et al., 2001; NASSAR et al., 2004), yet in women some cases showed intracytoplasmic pattern, two of them (67%) consisting of mixed type IMPC. Luna-Moré et

al. (2001) reported cytoplasmic pattern of EMA in different subtypes of breast carcinomas (lobular, papillary, NOS ductal, mucinous), but did not describe this staining in IMPCs. Thus, we postulate that the cytoplasmic pattern of EMA may be related to the mixed subtype of IMPCs.

According to literature women with IMPCs usually present dismal medium overall survival that range from 13 to 165.6 months that is associated with high mortality (MIDDLETON et al., 1999; PATERAKOS et al., 1999; TRESSERRA et al., 1998; ZEKIOGLU et al., 2004). Here we reported that up to 34 months of following period, only one woman died due to the disease. In comparative aspects, the following period of canine species was 404 days (that according to Lebeau (1953) represents 1616 days for human species) and 94% of the animals died due to the disease. Therefore it appears that the mortality in canine IMPCs is higher than in humans. It is noteworthy that the medium overall survival obtained for dogs, was also considered low when compared with another subtypes of mammary carcinomas in bitches, such as carcinomas in mixed tumours, that according to Rodrigues et al. (2015) might show about 1100 days medium overall survival.

## CONCLUSION

Taken together, human and canine IMPCs demonstrated to be aggressive neoplasms with high rates of lymph node metastasis and high proliferation index. Moreover these entities appear to show immunoprofiles related to well differentiated neoplasms. In comparative aspects canine IMPCs seems to be more aggressive neoplasms with larger tumour size, and higher rates of pure subtype, regional and distant metastasis and mortality, when compared to its human counterpart. Hence, canine mammary IMPCs appear to be a good spontaneous model which may be applied to better understand the biological behaviour of IMPCs in human species.

## CONFLICT OF INTEREST STATEMENT

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

ACKNOWLEDGEMENTS

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**Table 1.** Details of immunohistochemical reagents and methods used in the study. AR, antigen retrieval; IMPC, invasive micropapillary carcinoma; ER, estrogen receptor; PR, progesterone receptor; EMA, epithelial membrane antigen; HER-2, human epidermal growth factor receptor-2.

| Target antigen | Clone | Manufacturer | Dilution | AR Method | Incubation Time (h) |
|---|---|---|---|---|---|
| **Human IMPCs** | | | | | |
| **ER** | SP1 | Dako | Ready to use | Pressurised heating (125°C) | 18 |
| **PR** | PgR 636 | Dako | 1/300 | Pressurised heating (125°C) | 18 |
| **HER-2** | Polyclonal | Dako | 1/1500 | Pressurised heating (125°C) | 18 |
| **Ki-67** | MIB-1 | Dako | 1/200 | Pressurised heating (125°C) | 18 |
| **EMA** | E29 | BioGenex | 1/300 | Pressurised heating (125°C) | 18 |
| **Canine IMPCs** | | | | | |
| **ER** | 1D5 | Dako | 1:20 | Pressurised heating (125°C) | 1 |
| **PR** | HPRA2 | Neomarkers | 1:20 | Pressurised heating (125°C) | 1 |
| **HER-2** | Polyclonal | Dako | 1:200 | Water Bath (98°C) | 16 |
| **Ki-67** | MIB-1 | Dako | 1:25 | Pressurised heating (125°C) | 1 |
| **EMA** | E29 | Dako | 1:25 | Water Bath (98°C) | 16 |

**Table 2.** Clinicopathological and immunohistochemical features of IMPC in canine and human species. IMPC, invasive micropapillary carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor-2; EMA, epithelial membrane antigen.

| **Characteristics** | **Canine IMPC** | **Human IMPC** | **P** |
|---|---|---|---|
| Tumour size | | | |
| mean±SD | 5,94±3.31(13-0.50) | 2±1.80 (9-0.50) | 0.009 |
| (Max.-Min.) | | | |
| Lymph node metastasis | | | |
| Presence | 15(100) | 13 (54) | 0.007 |
| Absence | 0(0) | 11 (46) | |
| Distant metastasis | | | |
| Presence | 3 (30) | 0(0) | 0.005 |
| Absence | 7(70) | 25(100) | |
| Subtype of IMPC[a¶] | | | |
| 'Pure' | 14(70) | 8(32) | 0.01 |
| 'Mixed' | 6(30) | 17(68) | |
| Histological grade[b] | | | |
| I | 5(25) | 2(8) | 0.07 |
| II | 12(60) | 15(60) | |
| III | 3(15) | 8(32) | |
| Vessel invasion | | | |
| Absent | 4 (16) | 13 (52) | 0.03 |
| Present | 16 (64) | 12 (48) | |
| ER[c] | | | |
| + | 17(100) | 21(84) | 0.09 |
| - | 0(0) | 4(16) | |

| | | | |
|---|---|---|---|
| PR[c] | | | |
| + | 17(100) | 22 (88) | 0.15 |
| - | 0(0) | 3(12) | |
| HER-2[d] | | | |
| 0 | 7(35) | 11(44) | |
| 1+ | 8(40) | 9(36) | 0.03 |
| 2+ | 3(15) | 3(12) | |
| 3+ | 2(10) | 2(8) | |
| Ki-67[e] mean±SD (Max.-Min.) (%) | 12.10±8.7(29-1.7) | 30.9±21.6(80-5) | 0.0008 |
| EMA | | | |
| Inverted | 20 (100) | 18(85.7) | 0.08 |
| Cytoplasmic | 0 (0) | 3(14.3) | |

Some data were not available for all human and canine IMPCs. [a] 'Pure' subtype: carcinomas with a ≥75% infiltrating micropapillary pattern; 'mixed' subtype: carcinomas with a <75% infiltrating micropapillary pattern, associated with other infiltrating carcinomas; [b] Histological grading based on that of Elston and Ellis (1991, 1998); [c] '0', <1% nuclear staining in tumour cells; '+', >1% nuclear staining in tumour cells; [d] '0', no staining; 1+, weak, incomplete membrane staining of any proportion of tumour cells; 2+, complete membrane staining that is either non-uniform or weak in intensity, but with obvious circumferential distribution in 10% of cells, or intense, complete membrane staining of <30% of tumour cells; 3+, uniform, intense membrane staining of >30% of tumour cells. [e] number of nuclei positive for MIB-1 (anti-Ki-67) staining in a total of 1000 neoplastic cells from each lesion.

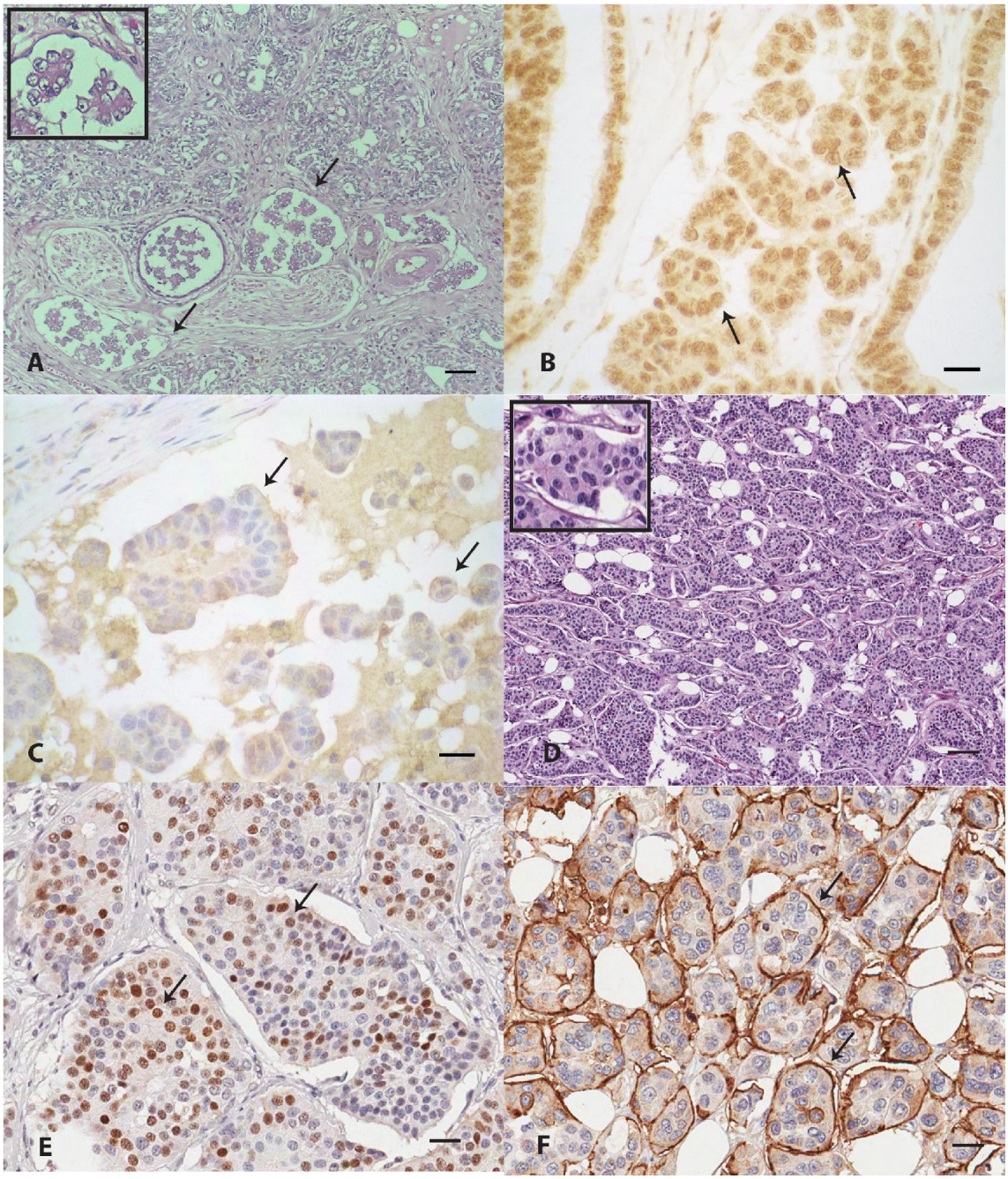



**Figure Legends**

**Figure 1. Photomicrographs illustrating features of invasive micropapillary carcinomas (IMPC) in canines: (A)** invasive areas of IMPC (arrows) (inset shows details) (HE stain, scale bar, 100 µm). **(B)** nuclear positivity for PR in invasive area (arrows) (Advance HRP Polymer Detection System, counterstained with Harris's haematoxylin, scale bar, 20 µm). **(C)** Invasive area showing reversed polarity pattern of EMA (arrows) (Advance HRP Polymer Detection System, counterstained with Harris's haematoxylin, scale bar, 20 µm); **and in humans: (D)** invasive areas of IMPC (inset shows details) (HE stain, scale bar, 100 µm); **(E)**

nuclear positivity for PR in invasive areas (arrows) (Novolink™ Polymer Detection System, counterstained with Harris's haematoxylin, scale bar, 40 μm). **(F)** Invasive areas showing reversed polarity pattern of EMA (arrows) (Novolink™ Polymer Detection System, counterstained with Harris's haematoxylin, scale bar, 40 μm).

10. ANEXOS

**ANEXO A - DECLARAÇÃO REFERENTE À REALIZAÇÃO DO EXAME DE QUALIFICAÇÃO.**

Faculdade de Medicina da UFMG

Universidade Federal de Minas Gerais
Centro de Pós-Graduação - CPG

**DECLARAÇÃO**

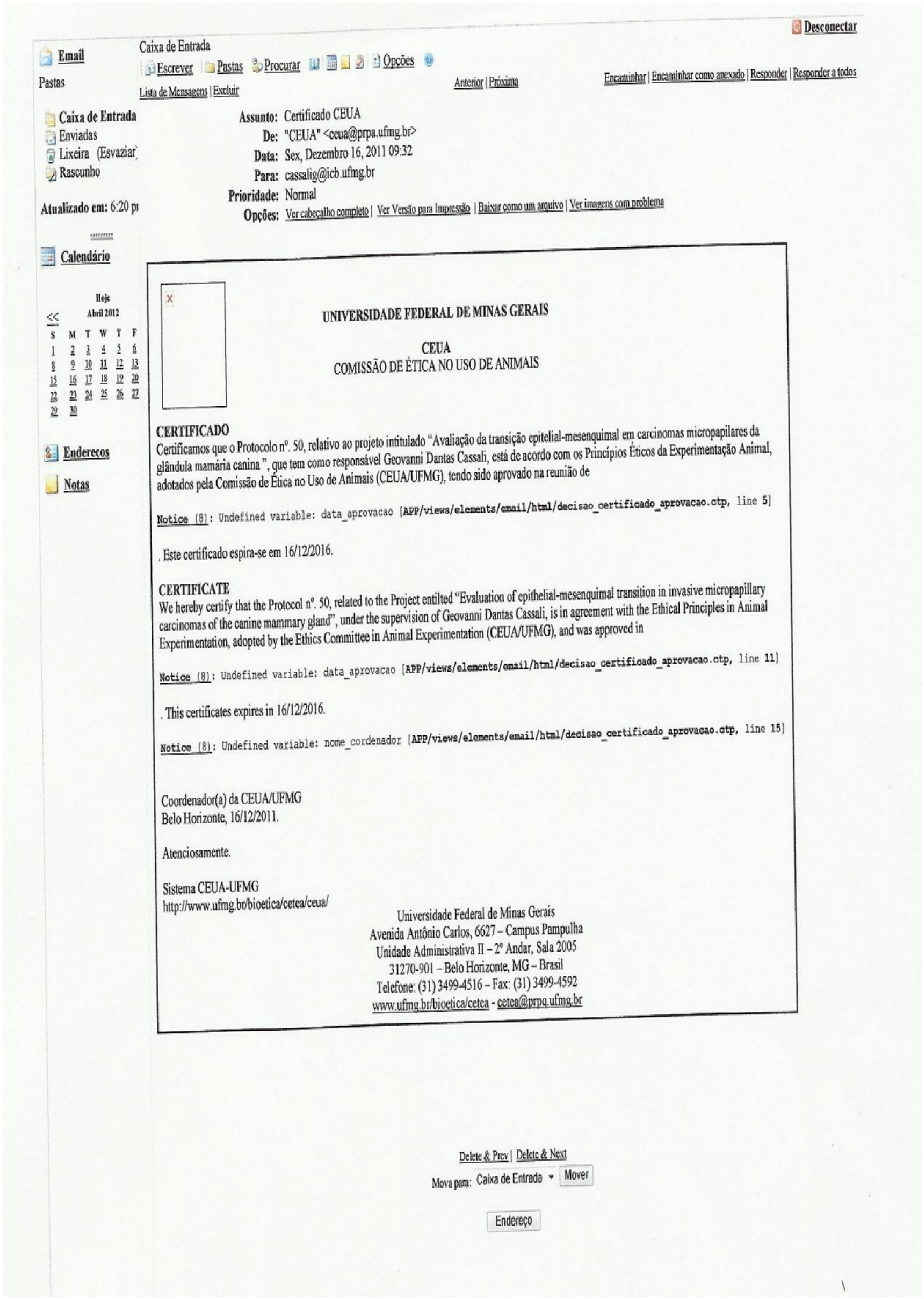
Declaramos, para fins curriculares, que **CONRADO DE OLIVEIRA GAMBA**, nº de registro 2012667150, integralizou os créditos exigidos do Curso de Pós Graduação em Patologia, nível doutorado, da Faculdade de Medicina da Universidade Federal de Minas Gerais, com defesa prevista para fevereiro de 2016. Declaramos ainda, que o aluno realizou o exame de qualificação, em 05/12/2014, sendo aprovado.

Belo Horizonte, 02 de setembro de 2015.

Centro de Pós-Graduação
Faculdade de Medicina / UFMG
Av. Prof. Alfredo Balena, 190 - 8º andar
CEP: 30130-100 - Funcionários - BH/MG

Líllian Oliveira
Centro de Pós-Graduação
Faculdade de Medicina - UFMG

**ANEXO B - CERTIFICADO DE APROVAÇÃO DO PROJETO DA TESE NO COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL – CETEA.**



Email
Pastas
Caixa de Entrada
Enviadas
Lixeira (Esvaziar)
Rascunho
Atualizado em: 6:20 pm
Calendário
Endereços
Notas

Desconectar
Caixa de Entrada
Escrever | Pastas | Procurar | Opções
Lista de Mensagens | Excluir
Anterior | Próxima
Encaminhar | Encaminhar como anexado | Responder | Responder a todos

Assunto: Certificado CEUA
De: "CEUA" <ceua@prpa.ufmg.br>
Data: Sex, Dezembro 16, 2011 09:32
Para: cassaliq@icb.ufmg.br
Prioridade: Normal
Opções: Ver cabeçalho completo | Ver Versão para Impressão | Baixar como um arquivo | Ver imagens com problema

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

**CERTIFICADO**

Certificamos que o Protocolo nº. 50, relativo ao projeto intitulado "Avaliação da transição epitelial-mesenquimal em carcinomas micropapilares da glândula mamária canina ", que tem como responsável Geovanni Dantas Cassali, está de acordo com os Princípios Éticos da Experimentação Animal, adotados pela Comissão de Ética no Uso de Animais (CEUA/UFMG), tendo sido aprovado na reunião de

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. Este certificado espira-se em 16/12/2016.

**CERTIFICATE**

We hereby certify that the Protocol nº. 50, related to the Project entitled "Evaluation of epithelial-mesenquimal transition in invasive micropapillary carcinomas of the canine mammary gland", under the supervision of Geovanni Dantas Cassali, is in agreement with the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation (CEUA/UFMG), and was approved in

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. This certificates expires in 16/12/2016.

Notice (8): Undefined variable: nome_cordenador [APP/views/elements/email/html/decisao_certificado_aprovacao.ctp, line 15]

Coordenador(a) da CEUA/UFMG
Belo Horizonte, 16/12/2011.

Atenciosamente.

Sistema CEUA-UFMG
http://www.ufmg.br/bioetica/cetea/ceua/

Universidade Federal de Minas Gerais
Avenida Antônio Carlos, 6627 – Campus Pampulha
Unidade Administrativa II – 2º Andar, Sala 2005
31270-901 – Belo Horizonte, MG – Brasil
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**ANEXO C - CERTIFICADO DO COMITÊ DE ÉTICA EM PESQUISA – COEP/UFMG REFERENTE AO PROJETO COMPARATIVO EXECUTADO COM RESULTADOS DESCRITOS NO APÊNDICE D EM FORMATO DE ARTIGO CIENTÍFICO.**

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM PESQUISA - COEP

**Projeto: CAAE – 25895013.8.0000.5149**

**Interessado(a): Prof. Geovanni Dantas Cassali**
**Departamento de Patologia Geral**
**Instituto de Ciências Biológicas - UFMG**

**DECISÃO**

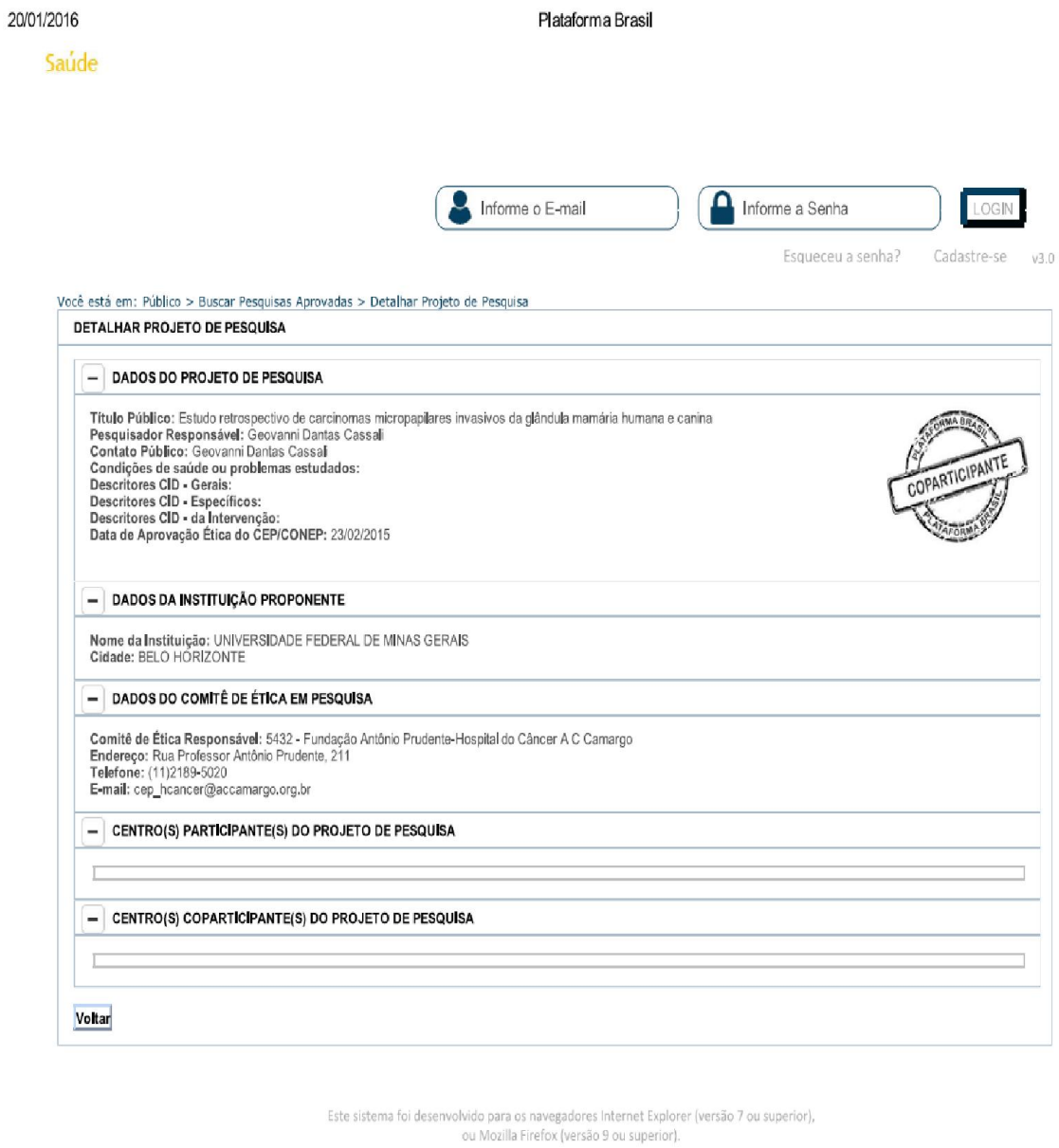
O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 16 de junho de 2014, o projeto de pesquisa intitulado **"Estudo retrospectivo de carcinomas micropapilares invasivos da glândula mamária humana e canina"**.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

**Profa. Maria Teresa Marques Amaral**
**Coordenadora do COEP-UFMG**

*Av. Pres. Antonio Carlos, 6627 – Unidade Administrativa II - 2° andar – Sala 2005 – Cep:31270-901 – BH-MG*
Telefax: (031) 3409-4592 - e-mail: coep@prpq.ufmg.br

**ANEXO D - CERTIFICADO DO COMITÊ DE ÉTICA EM PESQUISA – COEP/AC CAMARGO REFERENTE AO PROJETO COMPARATIVO EXECUTADO COM RESULTADOS DESCRITOS NO APÊNDICE D EM FORMATO DE ARTIGO CIENTÍFICO.**



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DETALHAR PROJETO DE PESQUISA

DADOS DO PROJETO DE PESQUISA

**Título Público:** Estudo retrospectivo de carcinomas micropapilares invasivos da glândula mamária humana e canina
**Pesquisador Responsável:** Geovanni Dantas Cassali
**Contato Público:** Geovanni Dantas Cassali
**Condições de saúde ou problemas estudados:**
**Descritores CID - Gerais:**
**Descritores CID - Específicos:**
**Descritores CID - da Intervenção:**
**Data de Aprovação Ética do CEP/CONEP:** 23/02/2015

PLATAFORMA BRASIL COPARTICIPANTE PLATAFORMA BRASIL

DADOS DA INSTITUIÇÃO PROPONENTE

**Nome da Instituição:** UNIVERSIDADE FEDERAL DE MINAS GERAIS
**Cidade:** BELO HORIZONTE

DADOS DO COMITÊ DE ÉTICA EM PESQUISA

**Comitê de Ética Responsável:** 5432 - Fundação Antônio Prudente-Hospital do Câncer A C Camargo
**Endereço:** Rua Professor Antônio Prudente, 211
**Telefone:** (11)2189-5020
**E-mail:** cep_hcancer@accamargo.org.br

CENTRO(S) PARTICIPANTE(S) DO PROJETO DE PESQUISA

CENTRO(S) COPARTICIPANTE(S) DO PROJETO DE PESQUISA

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