UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA DE BOTUCATU

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Perfil transcricional do câncer de mama em cadelas por sequenciamento de nova geração

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus de Botucatu, para obtenção do título de Mestra em Pesquisa e Desenvolvimento (Biotecnologia Médica).

Orientadora: Prof.ª Dr.ª Adriana Camargo Ferrasi
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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSANGELA APARECIDA LOBO-CRB 8/7500

Santos, Driéle Bretones dos.
Perfil transcricional do câncer de mama em cadelas por sequenciamento de nova geração / Driéle Bretones dos Santos. - Botucatu, 2022

Dissertação (mestrado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina de Botucatu
Orientador: Adriana Camargo Ferrasi
Coorientador: Geysson Javier Fernandez Garcia
Capes: 20804008

1. Cães - Doenças. 2. Carcinoma ductal de mama. 3. Transcriptoma.

Palavras-chave: Cadelas; Carcinoma ductal mamário; Transcriptoma.

Botucatu
2022

Aos meus pais pelo grande apoio e incentivo.

In memoriam a Kika.

Aos meus amigos pelo apoio emocional.

Agradecimentos

Os agradecimentos é uma das partes mais difíceis de escrever, porque não tem jeito certo. Eu agradeço a tudo e a todes, são tantas experiências, ocasiões, falhas, pessoas que ajudaram ou não, pois tudo isso definiu o que e quem sou hoje, o que eu faço e o que eu quero continuar fazendo.

Gostaria de manter meus agradecimentos também as pessoas que me ajudaram nas etapas anteriores na minha vida e foram mencionadas nos agradecimentos do TCC, mas vou destacar aquelas que se mantiveram e pessoas novas que surgiram nesses dois últimos anos, que foram difíceis.

Primeiro, eu gostaria de agradecer aos meus pais, Andréa e Rogério, pelo apoio e suporte na minha jornada acadêmica e na vida. Obrigada por tudo que vocês fazem e fizeram para que eu pudesse lutar um pouquinho pelo que eu quero ser e fazer da vida.

Agradeço aos meus amigos da graduação, Vivi, Cid, Carla, Rod e Pedro. Vocês são uma rede de apoio excepcional e ótima fonte de edificações. Infelizmente, nesses anos não pudemos estar tão presentes fisicamente, mas o apoio e o acolhimento sempre se mantiveram.

Agradeço aos meus amigos de infância, principalmente ao João Pedro. Uma pessoa de humanas que sempre acha incrível tudo que eu faço e está sempre lá para problematizarmos as coisas e para percebermos como somos duas chatas.

Agradeço as pessoas que a pandemia trouxe, especialmente a Tainá e as “pulgas”. Tainá, você fez esses anos menos ruins apenas ao estar do meu lado compartilhando vídeo aleatório e me ensinou muito. Quanto as “pulgas”, apesar dos pesares, o que eu sentia era legítimo e marcou o que eu sou também, por isso eu agradeço.

Agradeço as novas conexões de laboratório, em especial Alice, por todo companheirismo, ensinamento, edificações, entretenimento e música ruim; Profª Drª Fernanda Landim e Marina Landim, por acreditarem em mim e me ensinarem tanto.

Agradeço ao meu coorientador, Dr. Geysson Garcia, por ter paciência ao me ensinar e participar dessa jornada, tornando-a menos árdua.

Agradeço a minha orientadora, Profª Drª Adriana Ferrasi, por confiar em mim, por me acalmar e trabalhar na elaboração de um trabalho lindo. Obrigada pela dedicação, paciência e todo o conhecimento compartilhado. Espero que a senhora possa ter orgulho de mim, como eu tenho da senhora.

Agradeço as avaliadoras da banca, Profª Drª Estela Lima e Drª Marianna Rodrigues, por disponibilizarem tempo para contribuir com o trabalho. Tenho certeza de que com a participação de vocês esse trabalho será ainda melhor.

Agradeço aos meus professores da graduação, por despertarem em mim o gosto pela pesquisa e por abrirem diversas portas. Em especial, Profª Drª Kassandra Mustafé e Profª Drª Rejane Grotto.

Agradeço aos animais modelos, fundamentais para a realização desta pesquisa.

"*In ontologizing women and animals as objects, our language simultaneously eliminates the fact that someone else is acting as a subject/agent/perpetrator of violence*". (ADAMS, Carol J. Ecofeminism and the Eating of Animals. 1996, p.125).

RESUMO

SANTOS, D. B. **Perfil transcricional do câncer de mama em cadelas por sequenciamento de nova geração**. 2022. X p. Dissertação (Mestrado em Pesquisa e Desenvolvimento – Biotecnologia Médica) – Faculdade de Medicina de Botucatu, Universidade Estadual Paulista, Botucatu, 2022.

As neoplasias mamárias são frequentes em cadelas não castradas, representando até 46% dos casos de câncer nesses animais no Brasil. Cães podem ser um ótimo modelo de estudos sobre cânceres de mama já que estes animais e humanos compartilham características fisiológicas, histológicas e genômicas, além de apresentarem semelhanças quanto ao desenvolvimento, comportamento clínico e vias moleculares da doença. Análises comparativas do transcriptoma de tecidos tumorais e normais podem identificar genes e vias moleculares desreguladas, permitindo, a médio prazo, o desenvolvimento de ensaios laboratoriais para uso no diagnóstico, ou, a longo prazo, indicar potenciais alvos terapêuticos para medicina humana e canina. Nesse contexto, o objetivo principal do presente estudo foi, a partir do sequenciamento de nova geração (NGS), determinar o perfil transcricional de amostras de carcinomas ductais mamários em cadelas, buscando esclarecer a relevância dos transcritos desregulados nas vias moleculares envolvidas na doença. Para tanto, utilizou-se amostras de carcinoma ductal mamário e do tecido mamário não tumoral adjacente, provenientes da mastectomia radical de seis (6) cadelas, de diferentes raças e idades, atendidas pelo serviço de Cirurgia de Pequenos Animais do Hospital Veterinário da FMVZ-UNESP em Botucatu. O RNA total de cada amostra foi extraído, quantificado e submetido a análise de qualidade. Posteriormente, essas amostras foram reunidas em *pools* correspondendo a quatro grupos (dois tumorais e dois normais). Utilizou-se cada *pool* para a construção de uma biblioteca de cDNA, que, então foi sequenciada na plataforma *NextSeq 500 System* (Illumina, EUA). O resultado do sequenciamento foi encaminhado para análises de qualidade, limpeza e alinhamento ao genoma de referência, assim, identificando-se os transcritos diferencialmente expressos. Foram encontrados 11.278 genes dentre os, aproximadamente, 36.930 genes do genoma de referência de cachorro. Ao serem comparadas as amostras tumorais e o tecido normal adjacente, foram encontrados 1.206 genes diferencialmente expressos, sendo 633 regulados negativamente (downregulados) e 573 regulados positivamente (upregulados), os quais foram capazes de diferenciar os grupos pela PCA (*Principal Component Analysis*). A partir da análise de ontologia genética foram identificadas vias inflamatórias, de diferenciação e adesão celular e manutenção da matriz como as principalmente desreguladas nessa casuística. A maioria dos genes upregulados mais destacados estão envolvidos na organização da matriz extracelular, através da deposição de colágeno, vasculogênese mimética e da capacidade de invasão das células por meio da transição epitélio-mesenquimal. Ainda, os principais genes diferencialmente expressos observados no presente estudo, indicam maior agressividade da doença e pior prognóstico, destacando-se os dez genes mais upregulados. Finalmente, o estudo do transcriptoma canino parece indicar ser este um ótimo modelo para gerar informações relevantes à oncologia nas duas espécies.

Palavras-chave: Cadelas. Carcinoma ductal mamário. Transcriptoma.

ABSTRACT

Breast neoplasms are frequent in spayed female dogs, representing up to 46% of cancer cases in these animals in Brazil. Thus, dogs can be an excellent model for studies of breast cancer, because these animals and humans share physiological, histological, and genomic characteristics; in addition to having similarities in the development, clinical behavior, and molecular pathways of the disease. Therefore, comparative analyzes of the transcriptome of tumor and normal tissues can identify dysregulated genes and molecular pathways, allowing, in the medium term, the development of laboratory assays for use in the diagnosis, or, in the long term, indicate potential therapeutic targets for human and canine medicine. In this context, the main objective of this study was, from next-generation sequencing (NGS), to determine the transcriptional profile of samples of mammary ductal carcinomas in female dogs, seeking to clarify the relevance of deregulated transcripts in the molecular pathways involved in the disease. For this purpose, samples of tumor tissue from mammary ductal carcinoma and adjacent non-tumor breast tissue were used, from the radical mastectomy of 6 female dogs of different breeds and ages, attended by the Small Animal Surgery Service of the Veterinary Hospital of FMVZ- UNESP in Botucatu. The total RNA of each sample was extracted, quantified, and submitted to quality analysis. Subsequently, these samples were pooled corresponding to four groups (two tumoral and two normal). Each pool was used to build a cDNA library, which was then sequenced on the NextSeq 500 System platform (Illumina, USA). The sequencing result was sent for quality analysis, cleaning, and alignment with the reference genome, thus identifying the differentially expressed transcripts. 11,278 genes were found among approximately 36,930 genes in the reference dog genome. When comparing tumor and normal tissue samples, 1,206 genes were found to be differentially expressed, with 633 downregulated and 573 upregulated, which were able to differentiate the groups by PCA (Principal Component Analysis). From the analysis of gene ontology, inflammatory pathways, cell differentiation and adhesion, and matrix maintenance were identified to be mainly deregulated in this sample. Most of the most prominent upregulated genes are involved in the organization of the extracellular matrix, through collagen deposition, mimetic vasculogenic, and the invasiveness of cells through the epithelial-mesenchymal transition. Furthermore, the main differentially expressed genes observed in the present study indicate greater disease aggressiveness and a worse prognosis, highlighting the ten most upregulated genes. Finally, the study of the canine transcriptome seems to indicate that this is an excellent model to generate information relevant to oncology in both species.

Keywords: Female dogs. Ductal mammary cancer. Transcriptome.

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LISTA DE ABREVIATURAS E SIGLAS

5'UTR	5' *Untranslated Region*; Região 5' Não Traduzida
CEUA	Comissão de Ética no Uso de Animais
cDNA	*Complementar Deoxyribonucleic Acid*; Ácido Desoxirribonucleico Complementar
DEgenes	*Differentially Expressed Genes*; Genes Diferencialmente Expressos
DNA	*Deoxyribonucleic Acid*; Ácido Desoxirribonucleico
EMT	Epithelial-Mesenchymal Transition; Transição Epitélio Mesenquimal
GO	*Gene Ontology*; Ontologia Genética
GWAS	*Genome Wide Association Studies*; Estudo de Associação Genômica Ampla
HER2	*Human Epidermal Growth Factor Receptor 2*; Receptor-2 do Fator de Crescimento Epidérmico Humano
IGF	*Insulin-Like Growth Factor*; Fator de Crescimento Tipo Insulina
IL	Interleucina
mRNA	*Messenger Ribonucleic Acid*; Ácido Ribonucleico Mensageiro
NGS	*Next-Generation Sequencing*; Sequenciamento de Nova Geração
OMS	Organização Mundial da Saúde
PCA	*Principal Components Analysis*; Análise de Componentes Principais
RE	Receptores de Estrogênio
RNA	*Ribonucleic Acid*; Ácido Ribonucleico
RNAseq	Sequenciamento de RNA
RP	Receptores de Progesterona
SNPs	*Single Nucleotide Polymorphism*; Polimorfismo de Nucleotídeo Único
TGF-β1	*Transforming Growth Factor β*-1; Fator de transformação do crescimento β-1
TPM	*Transcripts Per Million*; Transcritos por Milhão

SUMÁRIO

CAPÍTULO 1

1. INTRODUÇÃO

Humanos e cães compartilham aspectos fisiológicos e genômicos. Assim, é comum que algumas doenças que acometem as duas espécies também apresentem semelhanças quanto a etiologia, desenvolvimento e comportamento clínico. Este é o caso de alguns cânceres mamários caninos e humanos, que partilham semelhanças biológicas e, muito provavelmente, vias moleculares. Por isso, cães podem atuar como um ótimo modelo de estudos desta doença.

O câncer é uma das principais causa de mortes de humanos e animais de companhia[1,2]. Em 2020, o câncer de mama foi o segundo maior responsável pelas mortes por câncer no mundo (contabilizando aproximadamente 685 mil mortes) e foi o mais incidente dentre os cânceres humanos (mais de 2,2 milhões de novos casos em 2020). Isso também é observado em animais de companhia, especialmente cães, visto que até 70% dos cânceres em cadelas não castradas são mamários, em sua maioria, malignos[3].

Devido à sua relevância, muitos estudos têm como foco os tumores mamários, principalmente os humanos, mas têm se expandindo para a espécie canina. Contudo, existem lacunas em nosso conhecimento sobre as vias moleculares envolvidas com a doença, dificultando o desenvolvimento de terapias mais eficazes e até mesmo a identificação de novos biomarcadores para diagnóstico precoce e/ou prognóstico. Nesse cenário, com o avanço das análises moleculares de alto rendimento, como o sequenciamento de nova geração (NGS), tem se tornado possível a caracterização do perfil da expressão gênica (transcricional) de tumores, quando comparado a sua contrapartida de tecido normal, por exemplo. Essas informações têm se mostrado valiosas para a compreensão das vias moleculares e redes regulatórias envolvidas nos mecanismos tumorais. Além disso, tais estudos permitem a identificação de moléculas biomarcadoras com potencial de uso em diagnóstico, prognóstico e até mesmo, como alvos terapêuticos em cânceres caninos e humanos.

Nesse contexto, o objetivo principal do presente estudo foi, a partir do sequenciamento de nova geração (NGS), determinar o perfil transcricional de amostras de carcinomas ductais mamários em cadelas, buscando esclarecer a relevância dos transcritos desregulados nas vias moleculares envolvidas na doença ao compará-los com dados de oncologia em humanos, já que ainda há lacunas no estudo transcriptômico do carcinoma mamário canino.

Este trabalho foi aprovado pela Comissão de Ética em Experimentação Animal (CEUA) como um subprojeto do estudo principal intitulado “Análise da ocorrência de metástase

intraocular e pulmonar em cadelas portadoras de carcinoma mamário e correlação com mutação e expressão de e-caderina", aprovado pela CEUA da Faculdade de Medicina Veterinária e Zootecnia – UNESP de Botucatu, sob protocolo número 49/2011.

O trabalho será apresentado em duas seções: i) uma breve revisão introdutória ao assunto e, ii) um artigo científico, já formatado segundo as normas do periódico escolhido para submissão (*Scientific Reports*; ISSN 2045-2322).

2. REVISÃO DE LITERATURA

Dentre os tipos de câncer que acometem cães não-castrados, o mais comum é o mamário[4]. Estudos epidemiológicos no Brasil mostraram que, entre 2004 e 2015, os cânceres mamários representavam de 32,6 a 44,58%[5–7] das neoplasias que acometem cães, variando conforme a região do pais. Além disso, a maioria dos casos são tumores malignos, totalizando, aproximadamente 70%[5,8]. Assim, os cânceres de mama são uma das principais causas de mortalidade de animais de estimação[1]. Esse alto risco de óbito está relacionado, principalmente, às metástases secundárias, que acometem, especialmente, pulmões e linfonodos[9].

As fêmeas adultas ou idosas são as mais afetadas pela doença, isto é, com idade entre sete e onze anos, variando conforme a região do país[5,7–10]. Essa idade é equivalente a idade média em que mulheres desenvolvem câncer de mama (entre 40 e 60 anos) pela escala de Lebeau[10]. Além da idade, outro fator de risco importante é a exposição hormonal[4,6]. Isso porque mudanças hormonais, como o ciclo estral e o uso de anticoncepcionais, estimulam o crescimento, assim como mudanças estruturais e funcionais das células da glândula mamária, como danos ao DNA e estímulo a mutagênese, angiogênese e proliferação dessas células, as quais podem ser iniciadas, prosseguindo a carcinogênese[4,12,13]. Nesse cenário, observa-se que a incidência é reduzida em regiões que realizam ovariohisterectomia das cadelas antes do primeiro ou segundo cio e, que a castração tardia, ou seja, após o segundo cio, não apresenta evidências de proteção efetiva conta o câncer de mama[4,9]. O risco de desenvolvimento da doença em cadelas ovariohisterectomizadas antes do primeiro ciclo estral é de 0,5%[9]. Por outro lado, o uso de contraceptivos hormonais, baseados em progesterona, estrógenos ou combinados, aumenta o risco de desenvolvimento do câncer[4].

Além disso, outros fatores podem aumentar os riscos como obesidade, dieta e raça[4,6]. Apesar de não ser determinante, estudos indicam que em cadelas castradas com massa corporal adequada até o primeiro ano de vida há redução do risco de desenvolvimento do câncer de mama em 99%, enquanto que em cadelas não castradas há redução de apenas 40%[14]. Ainda que o peso elevado não seja fator de risco isolado de desenvolver a doença[14], esse perfil corporal está relacionado ao desenvolvimento de cânceres mamários mais agressivos[15]. Isso também pode ser observado no câncer mamário humano, onde a ingestão de uma dieta mais calórica que o recomendado durante a puberdade pode levar a formação de lesões pré-neoplásicas, as quais podem se tornar malignas com estímulo hormonal[16]. Em humanos, a obesidade é um fator de risco para câncer mamário pós-menopausa, principalmente os tipos mais agressivos como o triplo negativo[13,17]. A obesidade afeta os níveis de adipocinas, hormônios esteroides, insulina e

fator de crescimento tipo insulina (IGF), além de alterar o microbioma e estimular processos inflamatórios[13].

No Brasil, as cadelas sem raça definida, poodles e pinschers são, respectivamente, as mais acometidas por câncer de mama[5,7,8]. Ainda assim, também pode haver influência da população de cães do país, já que estas são as raças mais comuns no Brasil.

Os tumores mamários caninos são muito heterogêneos quanto a morfologia e comportamento biológico[18] e vários sistemas de classificação já foram propostos. A Organização Mundial de Saúde (OMS) publicou, em 1974, a primeira Classificação Internacional Histológica de Tumores dos Animais Domésticos, que foi atualizada em 1999[19]. Recentemente, Cassali et al. (2011) e Goldschmidt et al. (2011) propuseram novas classificações para tumores mamários caninos, incluindo novos subtipos histológicos. A **Tabela 1** apresenta a classificação histológica proposta por Goldschmidt et al. (2011), amplamente utilizada atualmente.

Tabela 1 – Classificação Histológica de Tumores Desenvolvida por Goldschmidt et al.

NEOPLASIAS EPITELIAIS MALIGNAS	Carcinoma *in situ*	
	Carcinoma simples	Tubular
		Tubulopapilar
		Papilar cístico
		Cribriforme
	Carcinoma micropapilar invasivo	
	Carcinoma sólido	
	Comedocarcinoma	
	Carcinoma anaplásico	
	Carcinoma com desenvolvimento de adenoma complexo ou tumor misto	É detectável uma parte benigna na secção
	Carcinoma complexo	O componente epitelial é maligno e o mioepitelial é benigno
	Carcinoma misto	O componente epitelial é maligno, o componente mioepitelial mesenquimal é benigno e o componente mesenquimal é cartilaginoso ou ósseo.
	Carcinoma ductal maligno homólogo ao adenoma ductal	
	Carcinoma papilar intraductal maligno homólogo ao adenoma papilar intraductal	
NEOPLASIAS EPITELIAIS MALIGNAS ESPECIAIS	Carcinoma de células escamosas	
	Carcinoma adenoescamoso	
	Carcinoma mucinoso	
	Carcinoma rico em lipídeos	
	Carcinoma de células fusiformes	Mioepitelioma maligno
		Carcinoma de células fusiformes variantes de células escamosas
	Carcinoma inflamatório	
NEOPLASIAS EPITELIAIS MALIGNAS SARCOMAS	Osteosarcoma	
	Chondrosarcoma	
	Fibrosarcoma	
	Hemangiosarcoma	
	Outros	
CARCINOSARCOMA		
NEOPLASIAS BENIGNAS	Adenoma simples	
	Adenoma papilar intraducatal	
	Adenoma ductal	
	Fibroadenoma	
	Mioepitelioma	
	Adenoma complexo	
	Tumor misto benigno	
HIPERPLASIA E DISPLASIA	Ectasia ductal	
	Hiperplasia lobular	Regular
		Com atividade secretora
		Com fibrose interlobular
		Com atipia
	Epiteliose	
	Papilomatose	
	Alteração fibroadenomatosa	
	Ginecomastia	
NEOPLASIAS DO MAMILO	Adenoma	
	Carcinoma	
	Carcinoma com infiltração epidermal	
HIPERPLASIA E DISPLASIA DO MAMILO	Melanose da pele do mamilo	
HIPERPLASIA E DISPLASIA	Ectasia ductal	
	Hiperplasia lobular	Regular

Contudo, é inequívoco que a complexidade tumoral exige análises celulares e moleculares mais aprofundadas, como a classificação molecular disponível para os carcinomas mamários humanos. Nesse caso, resumidamente, a classificação molecular se dá conforme os seus perfis de expressão gênica e marcadores imunohistoquímicos, como o receptor-2 do fator de crescimento epidérmico humano (HER2), receptor de estrogênios (RE) e receptor de progesterona (RP). Os subtipos propostos são luminal A ($HER2^-$, ER^+ e RP^+), luminal B ($HER2^-/^+$, ER^+ e PR^+), HER2 ($HER2^+$, ER^- e PR^-) e triplo negativo ($HER2^-$, ER^- e PR^-)[20].

Os tumores do tipo luminal A apresentam maior expressão de genes epiteliais luminais e baixa expressão de Ki-67. Estes são de menor grau e, geralmente, tem bom prognóstico e respondem bem ao tratamento hormonal. Já os tumores do tipo luminal B são mais proliferativos, em razão da maior expressão do marcador Ki-67, e têm prognóstico intermediário, porém, pior que o do luminal A, principalmente quando positivo para HER2. Os tumores luminais são assim nomeados por possuírem a expressão semelhante as células luminais do epitélio da mama. O tipo HER2 apresenta prognóstico ruim associado a elevado índice proliferativo, no entanto o HER2 é um ótimo alvo para tratamento. Os tumores do tipo triplo negativo, na maioria das vezes, apresentam pior prognóstico e ainda não são conhecidas moléculas alvo para terapia efetiva[20].

Há tentativas de usar esta classificação para os cânceres de mama caninos, visto que a imuno-histoquímica destes marcadores teria valor diagnóstico, prognóstico e no tratamento, de forma mais acessível na medicina veterinária, sendo associada a outros métodos de avaliação[21]. Dentre os perfis moleculares, o mais comum em tumores de mama benignos caninos é o RE^+/RP^+, enquanto em tumores malignos é o RE^-/RP^+, visto que tumores mais agressivos tendem a ser independentes de hormônios, por isso o triplo negativo é aquele com pior prognóstico[22]. Além disso, tenta-se associar a classificação histopatológica aos subtipos moleculares em cães. O subtipo molecular HER2 foi correlacionado a todos os três tipos histológicos de câncer. Os subtipos $RE^+/HER2^+$ e triplo negativo mostraram correlação com carcinoma ductal. Já o subtipo RE^+ teve correlação com os subtipos histológicos simples e complexo[23].

Diagnóstico e estadiamento do câncer são essenciais para definir o tipo de tratamento que o paciente deve ser submetido e qual o prognóstico do animal, por exemplo a detecção de metástase a distância está associada a um prognóstico desfavorável[24].

Para o diagnóstico, é preciso fazer o exame clínico do animal por meio da avaliação da presença, tamanho e consistência de nódulos bem delimitados, formados a partir da proliferação

das células neoplásicas, avaliação dos linfonodos auxiliar e inguinal e percepção de ulceração na pele e inflamação local. É importante verificar se apenas uma mama está afetada, ou se há múltiplos tumores nas mamas, sendo que as mais afetadas são as mamas caudais abdominais e as inguinais[24] (**Figura 1**). Também, realiza-se a coleta de informações com o tutor, como histórico médico, ciclo reprodutivo, data de aparecimento da lesão e histórico de tumores anteriores. Além disso, a radiografia é uma técnica padrão, principalmente para verificar se há metástase pulmonar, por tratar-se do principal órgão distante invadido. Contudo, só é possível detectar lesões maiores que 6 mm, para lesões menores é utilizada a tomografia computadorizada[24]. Finalmente, o diagnóstico definitivo e o estadiamento do tumor será baseado no exame histopatológico[4].

O estadiamento é feito a partir do método TNM da OMS[25], que considera o tamanho do tumor (T), se afeta os linfonodos (N) e se existem metástases à distância (M), como descrito na Erro! Fonte de referência não encontrada.**2**. Entretanto, este método foi modificado em 1966, o nde o estadiamento depende unicamente do tamanho do tumor (por exemplo, T1 seria correspondente ao estágio I e consecutivamente), se não há invasão do linfonodo. Caso os linfonodos estejam envolvidos, o estágio é IV e, se apresenta metástase à distância, o estágio é V, independentemente do tamanho do tumor[4,9].

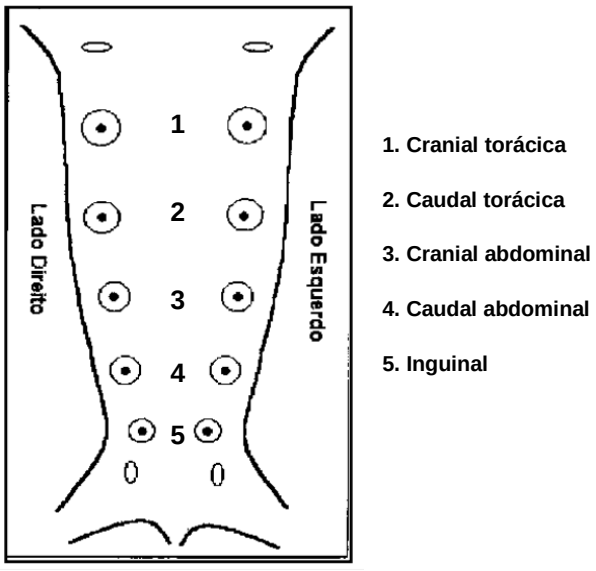

Figura 1 - Representação esquematizada das mamas de cadela. As mamas na região do tórax são denominadas torácicas, enquanto as localizadas no abdômen, são as abdominais. Sendo que as mamas voltadas a cabeça são chamadas craniais e aquelas voltadas para o rabo são as caudais (adaptado de PARDINI, 2015).

Tabela 2 - Métodos de estadiamento do câncer de mama para animais domésticos.

MÉTODO DESENVOLVIDO PELA OMS			
Tamanho do Tumor	Linfonodo Afetado	Metástase	Estágio
T_1	N_0	M_0	I
T_1	N_1	M_0	II
T_2	N_0	M_0	
T_3	N_0	M_0	III
$T_{1,2 \text{ ou } 3}$	$N_{0 \text{ ou } 1}$	M_1	IV
MÉTODO MODIFICADO POR MACEWEN E WITHROW			
T_1	N_0	M_0	I
T_2	N_0	M_0	II
T_3	N_0	M_0	III
$T_{1,2 \text{ ou } 3}$	N_1	M_0	IV
$T_{1,2 \text{ ou } 3}$	$N_{0 \text{ ou } 1}$	M_1	V

T_1 <3 cm. T_2 3 a 5 cm. T_3 > 5 cm. N_0: linfonodo não afetado. N_1: linfonodo afetado. M_0: sem metástase. M_1: com metástase. Adaptado de SORENMO et al. (2019).

O diagnóstico precoce é fundamental na redução da mortalidade por câncer. Atualmente, as técnicas utilizadas para o diagnóstico são por imagem (radiografia e ultrassonografia), exame histopatológico de biópsia do tecido afetado e testes de marcadores moleculares, principalmente imunohistoquímicos e bioquímicos.

Além do diagnóstico, o monitoramento do paciente durante e após o tratamento fornece informações relevantes para estimativas de prognóstico e possíveis intervenções terapêuticas em caso de recidivas da doença. Os avanços em biotecnologia têm proporcionado o desenvolvimento de métodos diagnósticos mais precoces e formas de monitoramento mais sensíveis e menos invasivas e, consequentemente, melhorando o prognóstico dos pacientes. Desse modo, análises do perfil de expressão gênica e mutações, especialmente dos oncogenes e supressores tumorais, podem discriminar os subtipos tumorais, assim como, indicar as vias mais promissoras para as terapias-alvo. Essa abordagem é mais comumente aplicada nos tumores mamários humanos, mas apresenta grande potencial também na medicina veterinária. Como por exemplo, determinar o perfil de expressão do oncogene HER2, pode predizer a progressão do câncer, indicar a melhor abordagem terapêutica e a provável resposta a ela[28]; e ainda, os perfis dos supressores tumorais *BRCA1*, E-caderina e p53, podem indicar o grau de agressividade do tumor, capacidade metastática, entre outras informações[26,29,30].

Buscando compreender as semelhanças entre as doenças nas duas espécies, alguns dos marcadores clássicos de câncer mamário humano já foram estudados em cães. BRCA1/2 são genes com múltiplas funções, incluindo o controle do ciclo celular e o reparo de quebras de fita dupla do DNA por meio de recombinação homóloga. Assim, mutações nesses genes predispõem seus portadores ao desenvolvimento de câncer, como o mamário[22]. As proteínas BRCA2 humanas e caninas apresentam 68% de homologia e, assim como em cânceres de mama humano, este gene é pouco transcrito no desenvolvimento de tumores de mama[22,31], enquanto é altamente expresso no tecido mamário normal[32]. Já BRCA1 apresenta expressão reduzida e deslocada de compartimentos celulares em carcinomas mamários. Foi observado que sua expressão no núcleo, onde exerce sua atividade, é reduzida e no citoplasma é aumentada em câncer de mama canino devido a mutações do gene, sendo que essa alteração pode estar relacionada a malignidade do tumor[33]. O gene p53 também é importante para a regulação do ciclo celular, assim, mutações nesse gene causam proliferação desregulada, levando a formação e progressão do tumor. Isso é bem documentado em tumores de mama em humanos[22]. Já em cães, identificou-se a alta expressão de p53, mutante ou não, como um marcador de prognóstico ruim e menor sobrevida[34,35].

Dessa forma, estudos baseados em análise do transcriptoma global de amostras tumorais podem revelar genes que possam ser utilizados como biomarcadores de risco ou prognóstico em câncer, sendo altamente específicos e, por vezes fáceis de detectar no próprio tecido e, muitas vezes circulantes no organismo, como no sangue e saliva[36,37]. Além disso, tais informações podem indicar vias moleculares desreguladas para o desenvolvimento de terapias alvo.

Em razão do alto grau de homologia entre o genoma canino e humano, as similaridades histopatológicas, biológicas e clínicas de alguns tumores nas duas espécies, a ocorrência natural do tumor nestes animais, mesmo com o sistema imune intacto, os cães são os melhores modelos comparativos para estudos sobre o câncer, sendo muito superiores à utilização de modelos animais com modificações induzidas ou cultura celular[22,38,39]. Outras características comuns de câncer de mama humano que podem ser observadas espontaneamente em cadelas são heterogeneidade dentro do tumor, desenvolvimento de resistência ao tratamento e presença de metástases a distância[39].

Vale reforçar que tais estudos representam benefícios às duas espécies, já que os resultados, quando ponderados adequadamente, podem ser extrapolados do modelo canino para o humano, em uma abordagem de oncologia comparativa. Ainda, os métodos diagnósticos,

prognósticos e terapêuticos que resultarem do conhecimento gerado por esse tipo de estudo poderá contribuir para avanços na medicina veterinária e humana.

3. OBJETIVOS

Definir o perfil transcricional do carcinoma ductal mamário em cadelas, buscando esclarecer a função e influência das moléculas desreguladas nas vias moleculares envolvidas na doença.

Objetivos Específicos

✓ Sequenciar, por sequenciamento de nova geração (NGS), o transcriptoma global de amostras de carcinomas ductais mamários caninos e de tecido mamário não tumoral;

✓ Identificar os transcritos com expressão diferencial em amostras de carcinomas ductais mamários caninos quando comparados ao tecido normal;

✓ Caracterizar os transcritos mais relevantes e avaliar o seu potencial como biomarcadores de diagnóstico ou prognóstico utilizando bancos de dados humanos.

CAPÍTULO 2

Artigo submetido na revista Scientific Reports

Title:

Transcriptomic profile of canine mammary ductal carcinoma

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Abstract

Dogs can be excellent models for spontaneous studies about breast cancers because presenting similarities in clinical behavior and molecular pathways of the disease. Thus, analyses of the canine transcriptome can identify genes and pathways deregulated, contributing to the identification of biomarkers e new therapeutic targets, benefiting humans and animals. In this context, the aim of the present study was to determine the transcriptional profile of canine mammary ductal carcinoma and contribute to the clarification of the importance of deregulated molecules in the molecular pathways involved in the disease. To this end, we used mammary ductal carcinoma tissue samples and non-tumor mammary tissue from radical mastectomy of six female dogs. Sequencing was performed on the NextSeq-500 System platform. When comparing carcinoma tissue and normal tissue were found 633 down and 573 upregulated genes, which were able to differentiate the groups by PCA. Gene ontology analysis indicated that inflammatory, cell differentiation and adhesion, and extracellular matrix maintenance pathways were mainly deregulated in this series. The main differentially expressed genes observed in this research indicate greater disease aggressiveness and worse prognosis. Finally, the study of the canine transcriptome appoint that this is an excellent model to generate information relevant to oncology in both species.

Keywords: dogs, mammary ductal carcinoma, transcriptome

1. Introduction

Cancer is the leading cause of death in humans and their domestic companions [1,2]. Breast carcinoma was the second most incident (2.2 million new cases) and caused 685,000 human deaths in 2020 [2]. Something similar can be observed among companion animals, especially dogs, since most tumors in uncastrated female dogs are mammary [3,4], with 50 to 75% being malignant [4].

Canine and human mammary cancers share several biological characteristics, such as histology, pathophysiology, clinical behavior, and molecular pathways [5,6]. Genomic analyses in canine tumors have revealed similarities between the two species, allowing new insight into the tumor's genetic basis [7]. Interestingly, several genes linked to increased risk of human cancers have also been observed in canines, such as mutations in BRCA1/2 [8-10] that predispose to breast and ovarian cancer in humans. This scenario presented above, combined with the shared home environment, makes modern dogs a suitable model for comparative oncogenetics studies.

Despite the relevance of the disease and the available studies, there are still gaps to be filled in the molecular mechanisms involved in canine mammary carcinoma. Thus, global transcriptomic research based on next-generation sequencing may contribute to a better understanding of the origins and progression of the tumor, besides contributing to the identification of biomarkers with potential use in the diagnosis, prognosis, and even as new therapeutic targets in cancer, benefiting humans and animals.

In this context, the aim of the present study was, based on next-generation sequencing, to determine the transcriptional profile of canine mammary ductal carcinoma samples and contribute to the clarification of the importance of deregulated molecules in the molecular pathways involved in the disease.

2. Casuistry and Methodology

2.1 Casuistry

We used mammary ductal carcinoma tissue samples and adjacent non-tumoral mammary tissue from radical mastectomy of 6 female dogs to build the sequencing libraries. The female dogs are non-castrated, of different breeds, aged 5 to 14 years, collected in Veterinary Surgery and Anesthesiology Service, São Paulo State University (UNESP), Botucatu, SP, Brazil.

2.2 Ethical Premises

This study was approved by the Animal Ethics Committee of FMVZ, UNESP, Botucatu, SP, Brazil (protocol 49/2011-CEUA).

2.3 Methodology

Sequencing: The tissue was preserved in RNAlater at -70°C until processing. 50 mg sample of the tissue was triturated using Precellys homogenizer® 24-Dual (Bertin Technologies, Rockville, Washington DC, USA). W extracted Total RNA of the samples using TRIzol (Thermo Scientific, USA). Then, RNA was quantified using Qubit RNA Assay Kit on Qubit 2.0 Fluorometer (Thermo Scientific, USA) and integrity (RIN > 8) was confirmed on Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). Subsequently, this material was

pooled, each with three samples, forming four groups (two tumoral and two normal). We used each pool to build the four sequencing libraries. The cDNA libraries were built with TruSeq™ Stranded Total RNA Sample Preparation Kit v2 (Illumina), and sequencing occurred on the NextSeq 500 System platform (Illumina, USA) using the NextSeq 500/550 High Output v2 150 cycles (Illumina) Sequencing Kit, according to the manufacturer's instructions.

Statistical Analysis: First, the data obtained from sequencing were subjected to quality and cleanliness analysis. The minimum Phred score was 32 per position in the alignment. Then, we mapped the paired-end reads to the dog reference genome using TopHat2 with the parameters: mate-inner-dist 200; mate-std-dev 100; no-novel-juncs; min-intron-length 40. The number of counts was obtained using HTSeq with default settings and normalized by DESEq2 (version 1.26). Significantly differentially expressed genes were considered those with $p<0.05$ and $|\log2 \text{ fold change}| \geq 1$. The abundance of mRNA transcripts was determined by converting transcripts per one million base pairs of mapped reads (TPM - Transcripts Per Million).

Gene Ontology Enrichment (GO): Performed in the Enrichr (https://maayanlab.cloud/Enrichr/) [11] platform, based on the hypergeometric test with Benjamini-Hochberg correction. We considered those enriched processes with $p<0.05$.

Identification of Transcription Factors: Using the X2K Web platform (https://maayanlab.cloud/X2K/) [12] was possible to identify the upstream regulation network of upregulated and down-regulated genes separately, thus indicating the transcription factors that regulate such sets of genes. In addition, we distinguished which differentially expressed genes are encoding transcription factors by comparing them to The Human Protein Atlas transcription factor database (www.proteinatlas.org) [13].

Identification of the Secretome: From The Human Protein Atlas database of secreted proteins, it was possible to identify which differentially expressed genes encode blood secreted proteins, which could be potential non-invasive biomarkers.

3. Results and discussion

We obtained four sequencing libraries, two tumoral libraries (T1 and T2) from tissues histologically classified as ductal breast carcinoma [14], and two control libraries (C1 and C2) from adjacent non-tumor tissue. All libraries were composed of a pool of samples from three patients with early-stage disease (T1N0M0). The mean age of the animals in the first sequencing was 10.0 years, and in the second sequencing was 7.7 years, with an overall mean of 8.9 years. This result agrees with epidemiological studies, which indicate that age is one of the main risk factors for the development of breast cancer [3]. From 8.0 years of age, this risk starts to be significant and increases with age [15].

Through global transcriptome analysis, we identified 11,278 genes among the 36,930 genes in the dog's reference genome (RefSeq) [16]. Comparing breast carcinoma and non-tumoral tissue samples, 1,206

differentially expressed genes (DEgenes) were found with statistical significance ($p<0.05$ and $-1 \geq \log2$ fold change ≤ 1), wherein 633 were negatively regulated (down-regulated) and 573 were positively regulated (upregulated) (Figure 1A-D). This transcriptional profile could distinguish each group by Principal Component Analysis (PCA) (Figure 1A), as in the heatmap (Figure 1B).

From the MA plot, which comprises the expression change level (log2 fold change) on the y-axis and the mean abundance (TPM - Transcripts Per Million) on the x-axis (Figure 2A), it was possible to identify abundant genes and genes with a high degree of deregulation (positive and negative). The most abundant genes showed lower levels of expression change, such as the upregulated gene CLU, which encodes a chaperone; SLPI, which encodes a protease inhibitor; and the downregulated TXNIP, a tumor suppressor that encodes a protein that protects the cell from oxidative stress. Most abundant genes ($TPM \geq 1000$) with a high degree of expression change ($-5 \geq \log2$ fold change ≥ 5) are down-regulated. The most upregulated genes, on the other hand, are less abundant ($TPM<100$), such as SERPINI2, which encodes a protease inhibitor protein; CDH12, despite its role in cell adhesion and proliferation, is not commonly studied in breast cancer; and WDR [17], a gene still little known. This MA plot profile is typical in cancers since the cell undergoes major changes, so highly upregulated genes are not common in that cell type, resulting in highly expressed genes that are not very abundant, the opposite being true, as shown in Figure 2A.

Also, in the MA plot, we highlighted the 10.0 most and 10.0 least expressed genes among those differentially expressed in the comparative analysis between the groups Mammary Carcinoma and Control. We observe that the down-regulated genes had log2 fold change <-7, in contrast to the upregulated genes had log2 fold change >5. Most of the upregulated genes are related to tissue organization, by encoding extracellular matrix component proteins (ACAN, VMO1, COL2A1, and COL8A1) and cell-cell (CDH12) and cell-extracellular matrix (CLEC3A) adhesion proteins. Also, some genes are involved in cell protection against oxidative stress (SERPINI2 and GPX2) and cell differentiation via the Wnt signaling pathway (SFRP2).

Most downregulated genes participate in muscle contraction by encoding skeletal and cardiac muscle components (TTN, MYLPF, TNNT3, MYL2, and MYL3) and the protein responsible for oxygen transport in these tissues (MB). In addition, some other down-regulated genes are associated with the expression of genes involved in lipid metabolism (FABD9), encoding the major milk protein (CSN1S1), and encoding an enzyme participating in mRNA editing (APOBEC2).

In the gene ontology (Figure 2B), 28 relevant pathways were identified, which act for tissue maintenance and cell cycle, being classified between cell support, migration, differentiation, proliferation, immune response, and ion regulation. In each pathway of the ontology was analyzed the percentage of up and down-regulated genes, differentiated by color, wherein red is the percentage of upregulated genes and blue is the percentage of down-regulated genes. Thus, we observed that the cell differentiation pathways have the most down-regulated genes, while the embryonic development pathway has the most upregulated genes. Furthermore, the most enriched pathways are related to cell adhesion, regulation of growth factor signaling, angiotensin signaling, and lactation.

The cell chemotaxis pathway was included in the immune response set because it represents other pathways for inducing positive chemotaxis of leukocytes, monocytes, and mast cells, which participate in the inflammatory response.

From the query of the DEgenes in X2K Web, the regulation network for the up and downregulated genes was defined separately. In this way, 98 transcription factors were found that regulate the set of upregulated genes and 95 that regulate the down-regulated genes. Next, the ten transcription factors that most regulate each group (up and downregulated genes) and their targets were highlighted, and the ontology of these targets was done (Figure 3). The ontology of the upregulated targets (Figure 3A) pointed out mainly immune, tissue organization, and hormonal pathways. Highlighting the negative regulation of IL-5 and IL-13 production, the response to IL-4, the response to chronic inflammation, and the differentiation of immune system cells (granulocytes and TCD4 and TCD8 lymphocytes). The tissue organization comprises the pathways of negative regulation of plasminogen activation, positive regulation of fibroblast migration, integrin-mediated regulation and cell adhesion, assembly of the β-catenin-TCF complex, and regulation of the epithelial-mesenchymal transition (EMT). The hormonal pathways are the most enriched. Among these, we observe the regulation of the metabolic process of hormones, prostaglandin transport, and cellular response to testosterone stimulation, in addition to the regulation of the IGF receptor signaling pathway.

In the ontology of down-regulated targets, we observe mainly pathways: tissue organization and cell adhesion, processes that participate in the cell cycle, immune pathways, and muscle cell activity. The regulation of the integrin biosynthesis process and integrin-mediated cell adhesion are the most enriched pathways and most associated with the transcription factors that regulate DEgenes.

It is also important to consider among the pathways of tissue organization the formation of the basement membrane, the negative regulation of cell leakage, and endothelial cell proliferation in the budding process of angiogenesis. The cell cycle processes range from the pyrimidine metabolic process, and calcium ion sensing to the IGF receptor signaling pathway. The immune pathways include negative regulation of T cell apoptosis, positive regulation of lymphocyte activation, and response to macrophage colony-stimulating factor. The pathways involved in muscle cell activity (actin-myosin filament sliding and sarcomere organization) are specific to the transcription factor MYOD1.

Interestingly, most of the highlighted transcription factors regulate upregulated and downregulated genes, such as SUZ12, NFE2L2, SMAD4, TP63, REST, and AR (which is part of the DESeq set). These transcription factors regulate the same functions between the groups (up and down-regulated genes), although through different targets and pathways.

Among the differentially expressed genes, we found 66 transcription factors (Figure 4A), of which 29 are upregulated (Figure 4B) and two were identified as regulators of the total set, and 37 are down-regulated (Figure 4C), with six being regulators of the DEgenes set, which is the case of the AR gene.

Based on the data from The Human Protein Atlas, a Venn diagram was drawn with the set of DEgenes, the total secreted protein genes and blood secreted protein genes data (Figure 5A). Thus, we identified 140 possible circulating protein genes that could be used as biomarkers. Among these genes, the ten most and ten least expressed genes in the MA plot were highlighted (Figure 5B).

From this set of genes that encode proteins secreted in the blood, the most expressed ones encode collagen (COL11A2 and COL8A1) but also stand out as the genes MMP7, which encodes a metalloproteinase responsible for the extracellular matrix breakdown for tissue remodeling; and LBP that encodes a protein that binds to Gram-negative bacteria wall lipopolysaccharides and regulates the immune response. In addition, some genes are not very abundant (TPM<10) but with high expressions, such as VSTM2A, which encodes a protein whose function is to regulate preadipocyte differentiation; and CNTN4, which encodes an axon-associated adhesion molecule.

On the other hand, the least expressed gene is ALB, which encodes albumin, already associated with a worse prognosis in women with breast cancer [17]. Also noteworthy are the genes SPP2, which encodes a protein of the cystatin superfamily; and CLEC3B, which encodes a lectin type C member 3 of family 3, which is associated with metastasis and tumor invasion [18] in other cancers, such as hepatocellular carcinoma. Moreover, there are very abundant genes (TPM>10000) but with low expression, as is the case of genes LUM, which encodes the lumican protein, which regulates the collagen fibrils organization; and COL3A1, which encodes the α1 chain of collagen type 3, found mainly in connective tissues of organs such as lung, uterus, and vascular system.

Gene ontology of the set of 140 genes encoding proteins secreted in the blood was performed (Figure 5C). In this analysis, 26 relevant pathways were identified, which are associated with immune response, extracellular matrix organization, angiogenesis, and cell cycle processes such as proliferation, migration, and cell death. We observed the signaling and activation pathways of immune system cells and chemotaxis of these cells as well as being enriched with only upregulated genes. The negative regulation pathway of plasminogen activation also comprises only upregulated genes and is one of the most enriched pathways. Other more enriched pathways are the response pathways to chronic inflammation and regulation of cytotoxicity of the complement system. However, the angiogenesis-related pathways were the least enriched.

The set of extracellular matrix organization pathways are well enriched and common to all the ontologies performed. It is possible to separate them into pathways related to cell adhesion, the organization of extracellular matrix components, and their degradation, which allows cell invasion.

Many genes highlighted in the MA plot are related to a poor prognosis in human breast cancer and other carcinomas, mainly by stimulating invasion capacity and metastasis at a distance and to lymph nodes. Those are the case with the high expression of CLU and SLPI, upregulated genes in the tumor samples (Figure 2A), which are correlated to cell migration and invasion capacity, favoring metastasis and thus resulting in more severe disease [19-21]. CLU encodes the protein isoform sCLU, which has chaperone activity, so it is overexpressed in stress situations to ensure the cell protection from apoptosis, through p53-dependent and p53-independent pathways. In addition, it participates in the extracellular matrix organization and the ease of cells' intravasation of blood and/or lymphatic vessels because it modulates the activity of MMP9 [22]. On the other hand, SLPI can prevent tissue destruction and regulates the inflammatory response [23]. Also, SLPI protein can induce vasculogenic mimicry [21], this is a type of angiogenesis characterized by the formation of microvascular channels composed of tumor cells, which feed the tumor and allow the perfusion of cells through the vessels [21,24].

Another interesting transcript is SERPINI2, which is overexpressed although not very abundant (Figure 2A). This gene encodes a myoepithelial serine proteinase inhibitor (MEPI), which is indirectly involved in reducing plasminogen activation. While negative regulation of plasminogen activation is very enriched. This plasminogen protection occurs in cells with high expression of SERPINI2, as observed in our results. Studies indicate that in breast tumors the expression of this gene is not observed, however with transfection of MEPI cDNA into breast neoplastic cells, inhibition of invasion is observed [25].

Besides these, it draws attention to the WDR49 transcript, encoded by a gene that is not commonly studied in breast cancer and with scarce information about its specific functions [26]. In addition, it participates in a family of genes that encode proteins regulating various cellular functions such as cell division and cell fate determination, ontology-enriched pathways, as well as transcription, transmembrane signaling, mRNA modification, and vesicle fusion [27,28]. However, HAN et al (2020) in a GWAS study of patients with hepatocellular carcinoma and chronic hepatitis B virus (HBV) infection reported low expression of WDR49 in these samples [29].

The proteolysis of the extracellular matrix, represented, for example, by the high expression of MMP7 (Figure 5B), is relevant for cell invasion and metastasis. However, excessive connective tissue formation is characteristic of human mammary ductal carcinoma [30]. And the density and organization of collagen fibrils can regulate the progression of mammary tumors in humans, mice, and dogs [31]. We observed high expression of several collagen types (COL2A1, COL8A1, COL11A1, COL11A2). These collagens can be used as myoepithelial markers and are involved in the progression of canine mammary ductal carcinoma in situ to invasive, mainly by activating the TGFβ signaling pathway, which is related to epithelial-mesenchymal transition (EMT), invasion, and stem cell characteristics [32-34]. In addition to these, other overexpressed genes in breast carcinoma samples related to EMT are SFRP2 [32-34], which modulates Wnt signaling - pathway highly enriched in ontologies that act on EMT among other tumor development processes [34]; and CDH12 [35,36], which encodes a type 2 N-cadherin from the family of proteins responsible for calcium-mediated cell adhesion. This function is associated with gene ontology, since the cell adhesion, homeostasis, and calcium transport pathways are highly enriched.

There is the involvement of transcription factors in EMT, such as the high expression of ZEB1 related to the high expression of CDH12 [35]. ZEB1 regulates upregulated genes in breast tumor tissue, however, this transcription factor is downregulated in the present study [Figure 4C). MECOM is one of the more upregulated genes encoding transcription factors. This transcription factor acts as a histone methyltransferase involved in several cellular functions regulation, such as cell proliferation [37]. MECOM is a stem cell transcription factor by regulates a set of cadherins [29]. Thus, high expression of MECOM is related to a higher risk of early death. Moreover, the higher the expression more advanced the stage of the disease [38].

ACAN is also involved in extracellular matrix remodeling and showed the highest fold change in the breast carcinoma samples. It encodes a proteoglycan specific to cartilaginous tissue, as does COL2A1, discussed earlier. There is evidence that the presence of these markers indicates metaplasia with cartilaginous characteristics [39,40]. In contrast, human mammary ductal carcinomas show no difference in ACAN

expression [41], which is understandable since metaplasia is uncommon in human breast cancers [39] but is frequent in benign tumors classified as mixed in dogs [42].

LUM encodes the lumican proteoglycan, a major component of the extracellular matrix, and plays an important role in angiogenesis, regulation of collagen fibrogenesis, cell invasion, and growth, which are important activities in tumor progression [33,40]. Its expression in breast cancer is controversial [40] but studies show that down-expression of LUM in human breast cancers represents a worse prognosis [41]. Such an expression profile can be observed in the results, where despite being abundant, LUM is downregulated.

Like LUM, the TXNIP is abundant but downregulated in the analyzed tumor samples. This gene encodes the thioredoxin binding protein, which from the binding with thioredoxin inhibits its function, causing the accumulation of reactive oxygen species, being then important for the control of oxidative stress of cells and, consequently, in the induction of apoptosis and inhibition of cell proliferation [43,44]. Thus, it can be considered a tumor suppressor, and its reduced expression is associated with several cancers, such as hepatocellular carcinoma, lung cancer, and breast cancer in humans [44], mice, and dogs [45]. Knock-down of the TXNIP gene in MCF-7 cells causes a reduction of the inhibitory effect on cell growth [45].

The most down-regulated genes are characteristic of muscle cells (MYL2, MYL3, MYLPF, TTN, TNNT3). In a transcriptional study of tissues from different types of canine mammary carcinoma, muscle-related pathways such as cardiac muscle morphogenesis, skeletal muscle adaptation, and muscle adaptation were enriched and associated with down-regulated genes in canine mammary ductal carcinoma tissues [46]. Similarly, several cell motility pathways and extracellular matrix organization were enriched and associated with upregulated genes [46]. Furthermore, these genes and proteins are found to be significantly less expressed in breast cancers [46,47] and other carcinomas, such as oral [48,49] and tongue squamous cell cancer [50], and neck and head squamous cell cancer-associated or not with smoking [51,52].

On the other hand, high expression of TTN and MYL3 genes can be described as a biomarker of worse prognosis in Ewing's sarcoma [53]. TTN was also more expressed in gastric adenocarcinoma [54]. TTN gene and the protein encoded by it are large, then more susceptible to mutations, which are related to muscle weakness, heart disease, muscular dystrophy, and breast cancer [55]. Single nucleotide polymorphisms (SNPs) in the TNN3 gene are associated with higher mammographic density, which is a risk factor for developing breast carcinoma [56]. The abundant but most down-regulated MB gene encodes the monomeric protein myoglobin, which is responsible for the transport and storage of O2 in muscle tissues. However, it has been shown that human breast epithelial cells also express this gene [57-60]. High expression of MB is observed in 40% of ductal carcinomas [59], however, there are significant cases of down-regulation of this gene in human breast cancer cells [60]. This indicates that MB may be a tumor suppressor associated with a better prognosis when upregulated, because the higher the expression, the less aggressive the disease [59]. High expression of MB is associated with lower cell proliferation by affecting the dynamics of mitochondria, causing mitochondrial hyperfusion, and preventing cell cycle progression from G1 to S phase [60]. Also, it indicates a change in the cellular energy matrix since the inactivation of the gene in vivo and in vitro leads to: increased O2 uptake, energy substrate switch from fatty acids to glycolysis (which may be associated with the downregulated FABP9 gene), therefore less O2 consumption, and increases the activity of mitochondrial

enzymes [57,59]. Although the molecular mechanisms related to MB are not well elucidated yet, this gene is commonly studied in breast and other hormone-dependent cancers such as prostate cancer because of its relationship with ERs (estrogen receptors). Just as long-lasting hypoxia leads to overexpression of the gene, the MB downregulation seems related to estrogen exposure [58,59]. One study evidenced that when breast tumor cells expressing RE (RE+) are exposed to β-estradiol, they showed lower expression of MB, possibly via regulation of RUNX1 [57], one of the transcription factors observed differentially expressed in the present study and which regulates the set of genes.

The genes that encode proteins secreted into the blood and that were mostly down and upregulated are ALB and LBP, respectively. Studies indicate that decreased serum albumin level, a protein encoded by the ALB gene, is related to shorter survival in breast cancer patients [17,61,62], while high levels reduced the risk of death by 45% [63]. This effect may be due to albumin's role in inhibiting estrogen-responsive tumor cell proliferation [64]. On the other hand, LBP encodes a lipopolysaccharide-binding protein, so it plays a role in the inflammatory response against bacteria by initiating the innate immune response. Therefore, it is related to inflammatory processes in the breast that lead to stromal remodeling and the development and progression of cancer in this tissue in humans [64] and mice [65]. Between these processes, the breast involution [65], which resembles the wound healing process, and the amyloidosis have been studied, with LBP showing amyloidogenic capacity [66].

This study pointed out the principal transcripts deregulated in canine mammary ductal carcinoma. As previously discussed, many of these genes already have been related to greater aggressiveness and worse prognosis in human and canine mammary carcinomas, mainly the ten most upregulated genes. Also, such alterations allowed us to infer the main deregulated pathways, which will contribute to a better understanding of the disease in dogs. Finally, the study of the canine transcriptome seems to indicate that this is an excellent model to discover relevant information to oncology in both species.

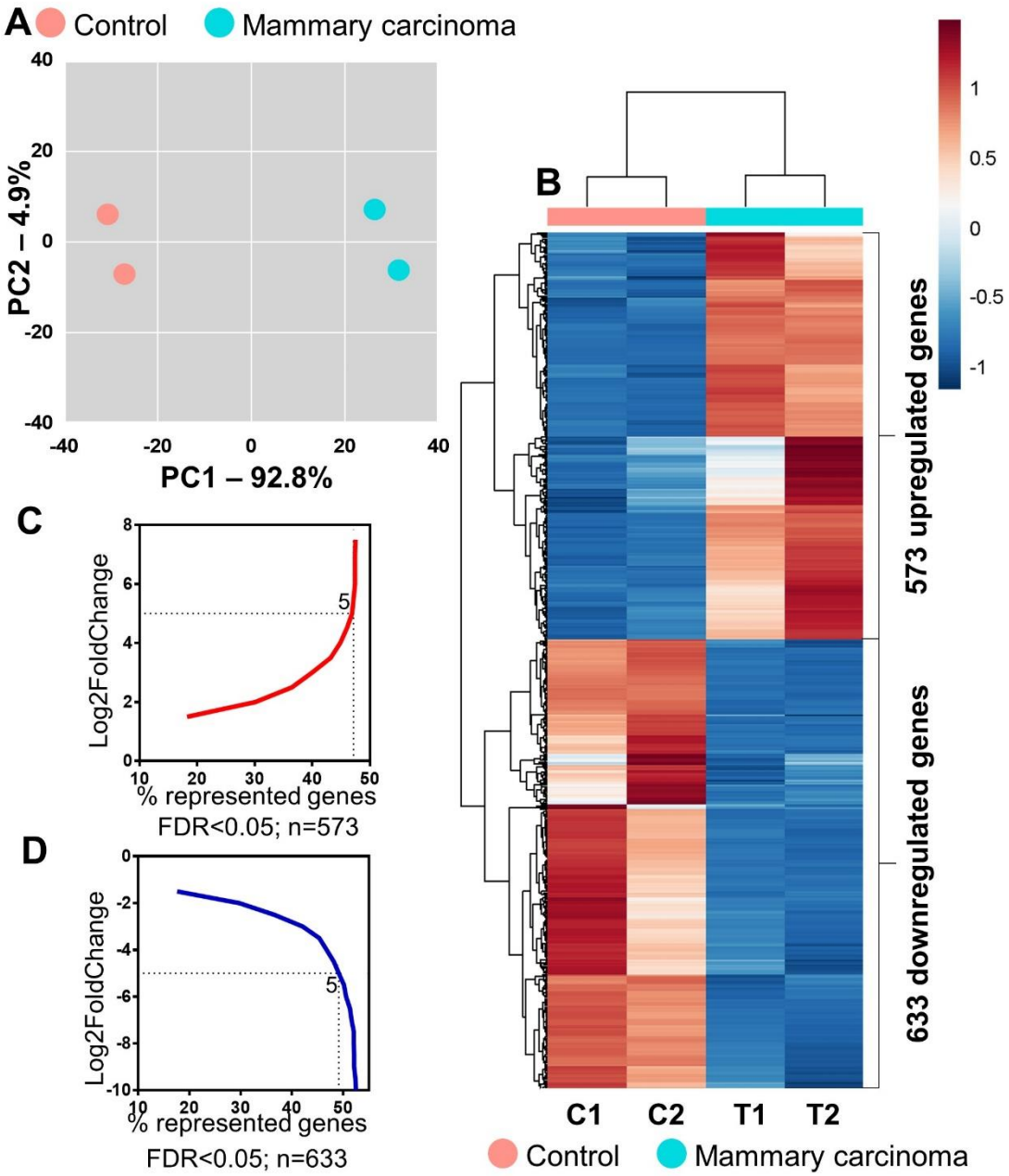

Figure 1 – Transcriptional analysis of mammary carcinoma samples from female dogs showed a differential gene expression profile. (A) Principal Component Analysis of the expression of the control (pink) and mammary carcinoma(green) genes showed a high percentage variance between the control and breast carcinoma groups (PC1=92.8%) but low within each group (PC2=4.9%). (B) Heatmap with the 1206 genes differentially expressed between the two groups: control (pink) and mammary carcinoma (green). (C) Graph of log2fold change distribution of upregulated (red) and (D) downregulated (blue) genes.

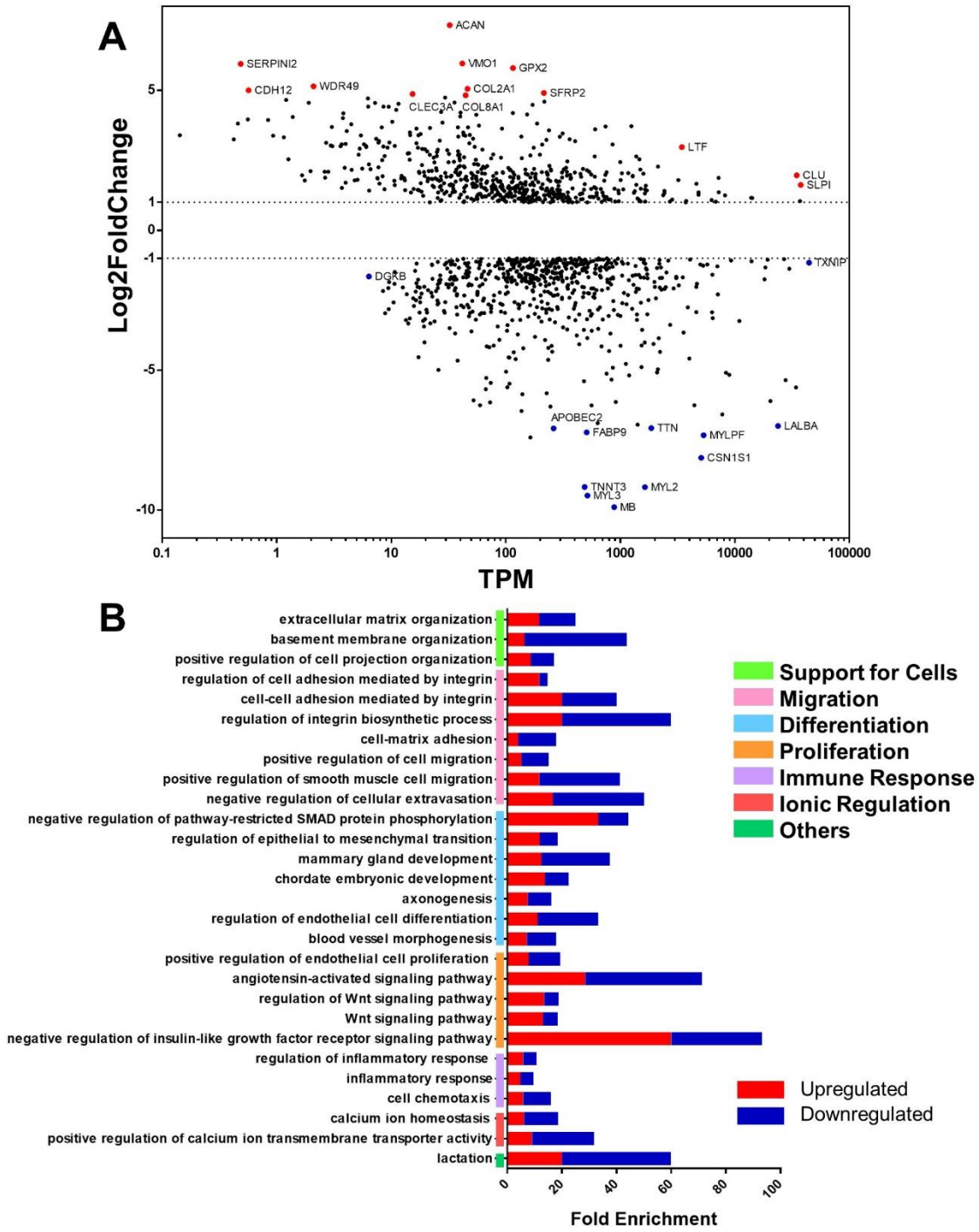

Figure 2 – Genes and cell pathways associated with mammary carcinoma in female dogs. (A) MA plot is a scatter plot that indicates the mean count abundance (TPM - Transcripts Per Million) and the degree of regulation (log2fold change). Genes with log2fold change>1 are overexpressed and genes with log2fold change<1 are underexpressed in the mammary carcinoma group when compared to normal tissue. The genes that showed greater abundance and a higher degree of positive regulation are highlighted in red. In blue the abundant genes with a high degree of negative regulation are highlighted. (B) Analysis of differentially gene enrichment in breast carcinoma, highlighting the main pathways affected. The colored bars indicate the percentage of upregulated (red) and downregulated (blue) genes among the total genes of each pathway.

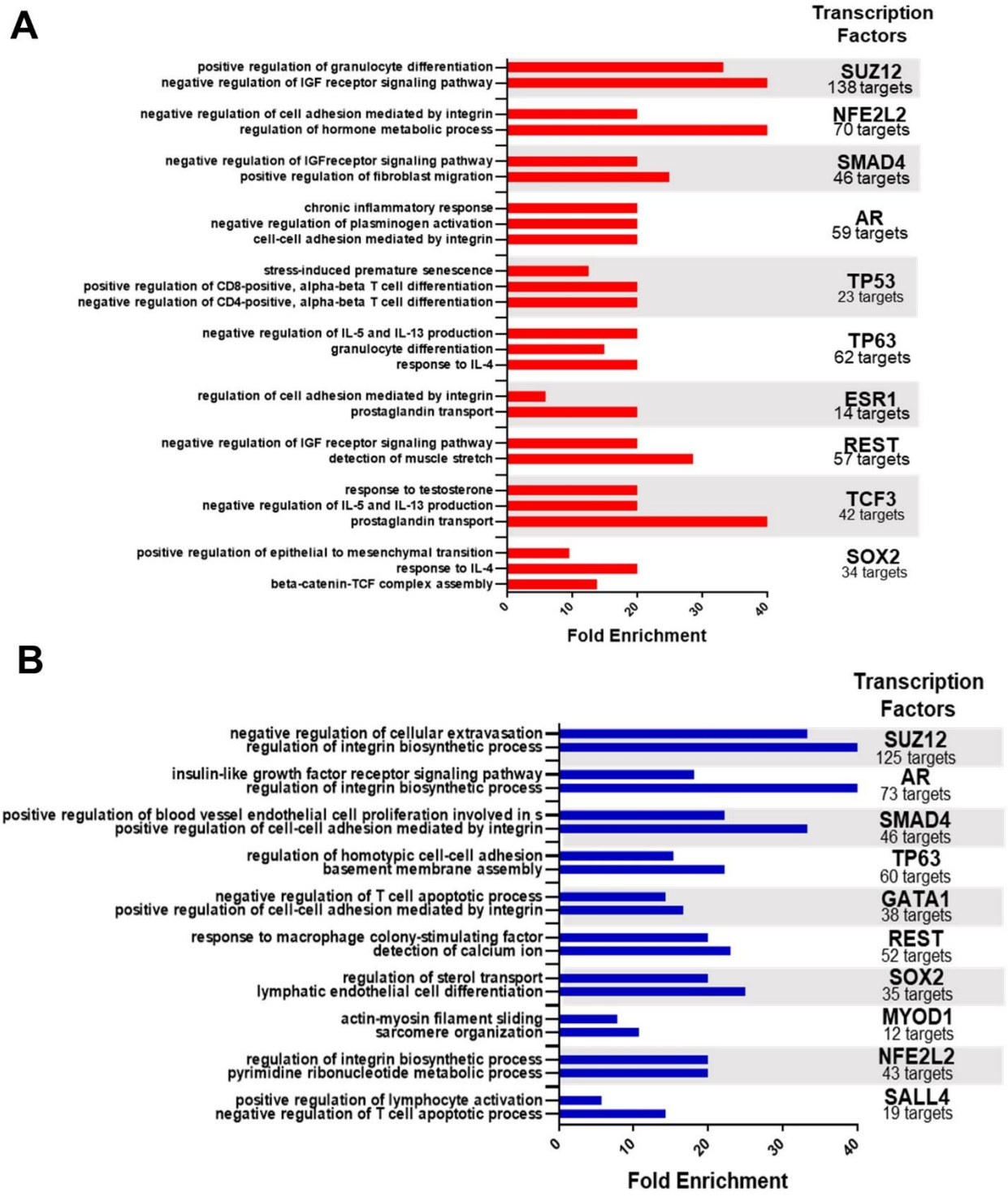

Figure 3 – Regulation pathways of differentially expressed transcription factors. (A) Enrichment analysis of target genes of the transcription factors that upregulate genes in mammary carcinoma. (B) Enrichment analysis of target genes of the transcription factors that downregulate genes in breast carcinoma. The main affected pathways of the main transcription factors and the number of targets is highlighted. The colored bars indicate the percentage of upregulated genes (red) and downregulated (blue) among the total genes of each pathway and on the right, the transcription factors that regulate the number of targets associated with the enriched pathways are highlighted.

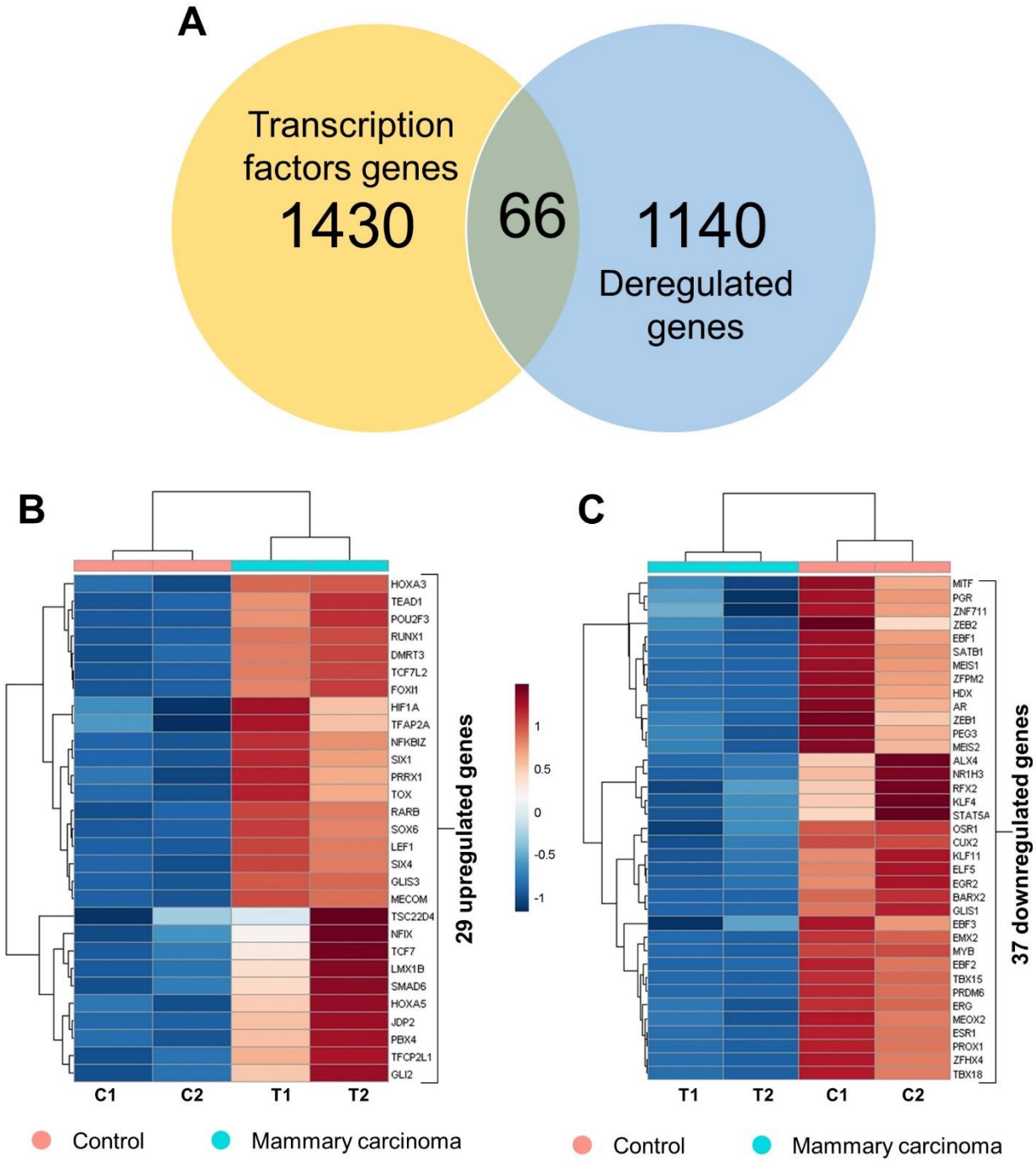

Figure 4 – Transcriptional analysis of mammary carcinoma samples from female dogs showed differentially expressed transcription factor genes. (A) Venn diagram discriminates the differentially expressed genes found by the analyzes performed and all the genes that encode transcription factors according to The Human Protein Atlas database. (B) Heatmap with the 29 genes of upregulated transcription factors in canine mammary carcinoma; control (in pink) and mammary carcinoma (in green). (C) Heatmap with the 37 transcription factor genes. Analysis of target genes, within the set, of the five main transcription factors that regulate upregulated genes and downregulated genes.

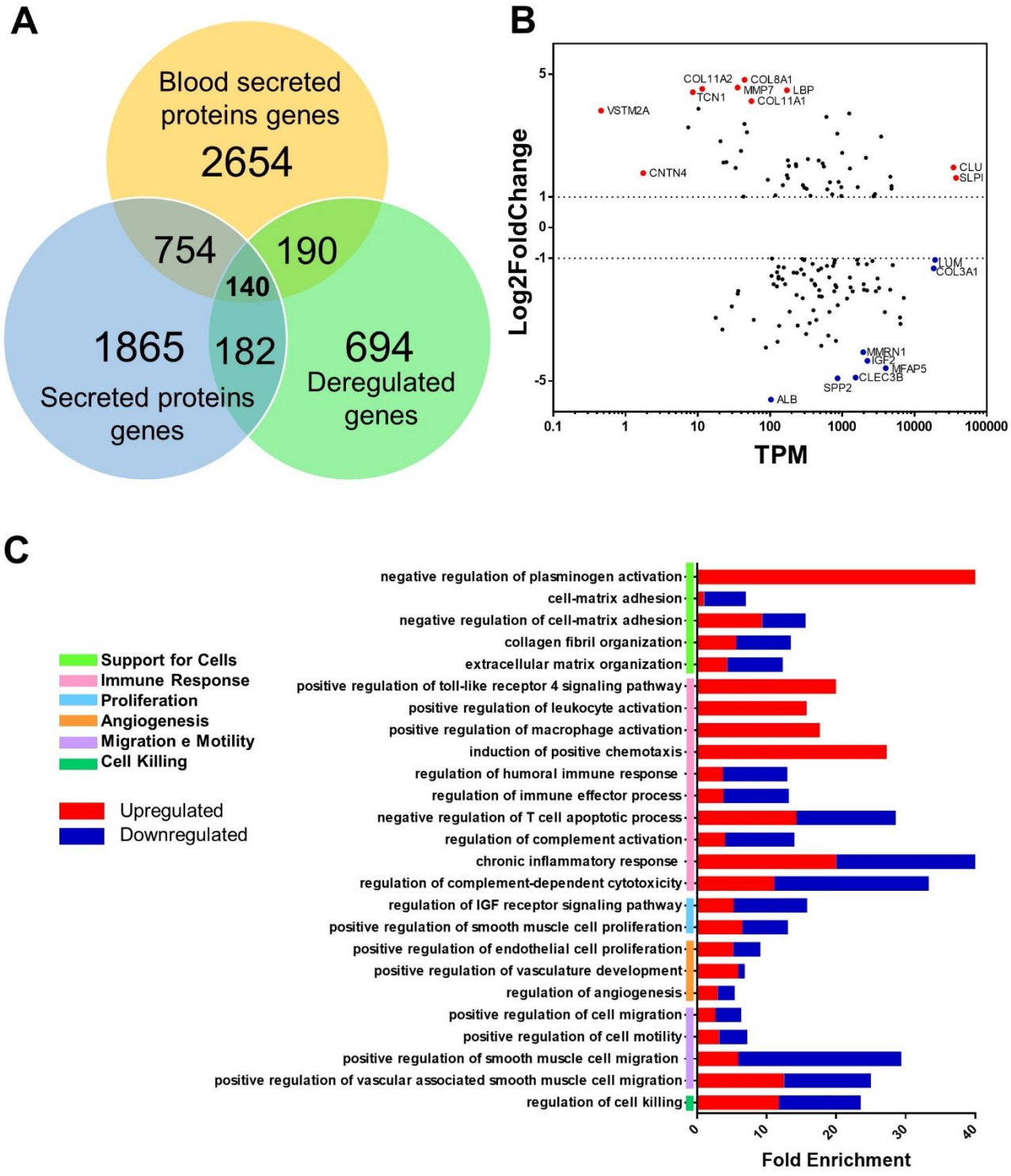

Figure 5 – Highlighting genes encoding secreted proteins and cellular pathways associated with mammary carcinoma in female dogs as potential biomarkers. (A) Venn diagram that discriminates the differentially expressed genes found by the analyzes performed, all the genes that encode secreted proteins, and all the genes that encode proteins specifically secreted in the blood according to The Human Protein Atlas database. (B) MA plot of the 140 genes that encode proteins secreted in the blood, with those genes with a higher degree of positive regulation being highlighted in red, and those genes with a high degree of negative regulation in blue. (C) Analysis of enrichment of genes encoding secreted blood proteins found differentially expressed in breast carcinoma, highlighting the main pathways affected. The colored bars indicate the percentage of up-regulated (red) and down-regulated (blue) genes among the total genes of each pathway.

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CONSIDERAÇÕES FINAIS

O presente estudo evidenciou a importância de análises transcriptômicas no modelo canino. Muitos dos genes diferencialmente expressos e vias moleculares identificadas tiveram o seu papel descrito através da abordagem de comparação com os dados já disponíveis para a espécie humana, destacando a relevância dos estudos comparativos entre espécies. Os resultados indicaram genes com potencial uso no prognóstico e alterações moleculares associadas ao carcinoma mamário ductal canino que são pouco exploradas como alvos terapêuticos e que merecem mais estudos.

Ademais, foram identificadas diversas relações de assinaturas de subtipos do carcinoma mamário (como marcadores mioepiteliais, marcadores de cartilagem, relação de regulação hormonal dos genes), e assim, a próxima etapa desse trabalho é verificar o quanto esse tipo de tumor poderia ser comparável a algum subtipo de carcinoma mamário humano.

Independente disso, o perfil transcricional do câncer de mama em cadelas em estágio inicial revelou várias alterações moleculares que levam a transformação celular, progressão e metástase, assim, indicando possíveis biomarcadores e alvos terapêuticos para essa doença em cadelas e mulheres.

Finalmente, o estudo do transcriptoma canino parece indicar ser este um ótimo modelo para gerar informações relevantes à oncologia nas duas espécies.

ANEXO

[MATERIAL SUPLEMENTAR]

Tabela 3 – Lista dos 10 genes menos e mais expressos entre os grupos controle e carcinoma mamário canino.

GENES MAIS EXPRESSOS

Gene	log2FoldChange	p-value	Função
ACAN	7,3279	3,9981E-147	Codifica um membro da família de proteoglicanos agrecanos/versicanos, o qual compõe a matriz extracelular do tecido cartilagionoso.
VMO1	5,9623	6,9011E-08	Codifica a proteína 1 da camada externa da membrana vitelina
SERPINI2	5,9477	1,9701E-06	Codifica uma proteína inibidora de proteases de serina, que participa da regulação de vários processos fisiológicos, como desenvolvimento, malignidade e inflamação.
GPX2	5,7979	1,4090E-49	Codifica a proteína glutationa peroxidase 2, responsável por proteger as células de danos de estresse oxidativo ao reduzir peróxido de hidrogênio.
WDR49	5,1457	1,4390E-29	Codifica um membro da família de repetição WD40, que é responsável pela regulação do controle do ciclo celular e apoptose.
COL2A1	5,0561	1,6920E-38	Codifica a protéina colágeno tipo 2 de cadeia α1, que é encontrado em cartilagem e no humor vítreo.
CDH12	5,0018	3,3303E-25	Codifica caderina 12, responsável por mediar a adesão celular dependente de cálcio.
SFRP2	4,9071	9,3678E-107	Codifica uma proteína da família SFRP, sendo responsável por modular a sinalização Wnt.
CLEC3A	4,8685	6,1953E-12	Codifica a proteína lectina tipo C membro A da família 3.
COL8A1	4,8230	5,0763E-173	Codifica a protéina colágeno tipo 8 de cadeia α1, que é encontrado na membrana basal do endotélio córneo.

GENES MENOS EXPRESSOS

Gene	log2FoldChange	p-value	Função
TTN	-7,0702	2,6755E-158	Codifica a proteína titina, que atua na adesão de proteínas que compõem os sarcômeros das células do músculo estriado e regulam o com o comprimento deste.
APOBEC2	-7,0771	1,0662E-08	Codifica a subunidade 2 da enzima catalítica de edição de mRNA apolipoproteían B
FABP9	-7,2249	8,3929E-27	Codifica a proteína 9 de ligação de ácidos graxos, que participa das vias de metabolismo de lipoproteínas.
MYLPF	-7,3219	2,4256E-29	Codifica a miosina de cadeia leve 2 (isoforma do músculo esquelético), responsável por ligar em íons de cálcio que inicia a contração.
ENSCAFG00000032415	-7,4008	1,7872E-10	Não foi encontrado
CSN1S1	-8,1279	4,3095E-115	Codifica a proteína caseína α1, componente do leite.

TNNT3	-9,1710	6,4862E-20	Codifica a proteína troponina 3, que compõe o complexo trimérico de toponina, o qual inicia a contração muscular.
MYL2	-9,1776	8,7293E-32	Codifica a miosina de cadeia leve 2 (isoforma do músculo cardíaco), responsável pela ligação em íons de cálcio que inicia a contração.
MYL3	-9,4833	4,3921E-10	Codifica a miosina de cadeia leve 2 (isoforma do músculo cardíaco e esquelético)
MB	-9,8947	2,1924E-17	Codifica uma mioglobina, muito encontrada no músculo cardíaco e esquelético.

Tabela 4 – Lista dos 10 genes codificantes de proteínas secretadas no sangue mais e menos expressos entre os grupos controle e carcinoma mamário canino.

GENES MAIS EXPRESSOS SECRETADOS NO SANGUE

Gene	log2FoldChange	p-value	Função
COL8A1	4,8230	5,0763E-173	Codifica a cadeia α1 do colágeno tipo 8, que é encontrado na membrana basal do endotélio córneo.
MM7	4,5682	1,8633E-23	Codifica a proteína metaloproteinase de matriz 7, responsável pela quebra da matriz extracelular em situações fisiológicas como, remodelamento do tecido e desenvolvimento embrionário.
COL11A2	4,5222	4,7112E-12	Codifica a cadeia α2 do colágeno tipo 11, que é responsável pelo controle do crescimento de fibrilas de colágeno na fibrinogênese.
LBP	4,4808	5,4152E-53	Codifica a proteína de ligação a lipopolisacarídeo, que a partir dessa ligação com o componente da parede de bactérias gram-negativas regula a resposta imunológica aguda.
TCN1	4,4181	1,2372E-05	Codifica uma proteína de ligação a vitamina B12, facilitando sua entrada nas células.
COL11A1	4,1254	7,6300E-147	Codifica a cadeia α1 do colágeno tipo 11, que é responsável pelo controle do crescimento de fibrilas de colágeno na fibrinogênese.
LRP2	3,8797	4,1508E-46	Codifica a proteína relacionada a lipoproteína de baixa densidade 2 (megalin), reponsável pela ligação a diversas macromoléculas, como albumina, lipoproteínas, vitaminas e hormônios, assim, também atuando na sinalização celular.
VSTM2A	3,8143	0,0032	Codifica a V-Set And Transmembrane Domain-Containing Protein, responsável por regular a diferenciação de pré-adipócitos em branco ou marrom.

PENK	3,7208	7,7418E-26	Codifica uma préproteína que ao ser quebrada resulta em peptídeos opioides que modulam a percepção de dor.
IGFBP5	3,6086	9,7333E-35	Codifica a proteína de ligação do fator de crescimento tipo insulina 5, regulando o efeito promotor de crescimento.

GENES MENOS EXPRESSOS SECRETADOS NO SANGUE

Gene	log2FoldChange	p-value	Função
TTN	-7,0702	2,6755E-158	Codifica a proteína titina, que atua na adesão de proteínas que compõem os sarcômeros das células do músculo estriado e regulam o com o comprimento deste.
APOBEC2	-7,0771	1,0662E-08	Codifica a subunidade 2 da enzima catalítica de edição de mRNA apolipoproteían B
FABP9	-7,2249	8,3929E-27	Codifica a proteína 9 de ligação de ácidos graxos, que participa das vias de metabolismo de lipoproteínas.
MYLPF	-7,3219	2,4256E-29	Codifica a miosina de cadeia leve 2 (isoforma do músculo esquelético), responsável por ligar em íons de cálcio que inicia a contração.
ENSCAFG00000032415	-7,4008	1,7872E-10	Não foi encontrado
CSN1S1	-8,1279	4,3095E-115	Codifica a proteína caseína α1, componente do leite.
TNNT3	-9,1710	6,4862E-20	Codifica a proteína troponina 3, que compõe o complexo trimérico de toponina, o qual inicia a contração muscular.
MYL2	-9,1776	8,7293E-32	Codifica a miosina de cadeia leve 2 (isoforma do músculo cardíaco), responsável pela ligação em íons de cálcio que inicia a contração.
MYL3	-9,4833	4,3921E-10	Codifica a miosina de cadeia leve 2 (isoforma do músculo cardíaco e esquelético)
MB	-9,8947	2,1924E-17	Codifica uma mioglobina, muito encontrada no músculo cardíaco e esquelético.

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