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ADÁLIA FREITAS DE OLIVEIRA LOPES

GLOBAL PROTEOMIC ANALYSIS OF TUMOR CELLS AND STROMAL FROM CANINE MAMMARY TUMORS: A POTENTIAL MODEL FOR COMPARATIVE ONCOLOGY

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Dissertation presented to Graduation Program in Animal Science of the Federal University of Ceará as partial fulfillment of the requirements for the degree of Master in Animal Science. Concentration area: Ruminant Production, Forage and Pasture.

Advisor: Prof. Ph.D. Arlindo de Alencar Araripe Noronha Moura.
Co-advisor: Ph.D. Denise Damasceno Guerreiro

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RESUMO

A presente dissertação teve dois objetivos: 1) discutir o valor de análises específicas em TMC usando transcriptômica e proteômica para a oncologia comparativa através de uma revisão bibliográfica; 2) determinar uma análise comparativa e quantitativa de proteínas provenientes de TMC usando a abordagem proteômica bottom-up. No capítulo 1 foi abordado a importância do uso das cadelas com tumores mamários espontâneos como modelo animal para o câncer de mama humano. O estudo de TMC pode contribuir fortemente para a compreensão do curso da doença e contribuir para a descoberta de biomarcadores não só de diagnóstico, como também de biomarcadores prognósticos e terapêuticos do câncer de mama. A expressão de genes e proteínas de tumores através do uso de análises como transcriptômica e proteômica de células tumorais presentes em TMC forneceram uma riqueza de dados sobre a expressão genética, porém o estroma tumoral carece de estudos. No estudo 2 buscamos candidatos a biomarcadores de estado tumoral mamário através da abordagem proteômica. Comparamos a expressão de proteínas de tumores mamários caninos (TMC) e glândulas mamárias normais (GN). Foram coletadas amostras de tecidos (5 TMC e 5 GN) obtidas de cadelas post-mortem do Centro de Controle de Zoonoses (CCZ). As amostras foram divididas em duas porções. Uma porção foi congelada e armazenada em -17 ◦C até ser utilizada para análise proteômica e a outra porção seguiu para os procedimentos padrão de biópsia e foi armazenada à temperatura ambiente. Tumores mistos benignos (n=2), carcinoma inflamatório (n=1), adenomas complexos (n=2), NT (n=5) foram selecionados para a abordagem proteômica em shotgun. A pesquisa de espectro peptídico foi realizada usando Progenesis v4.2 no qual permitiu a identificação em média de 945 proteínas, sendo 136 proteínas super expressas em TMC. A maioria dessas proteínas super expressas estava envolvida em processos biológicos relacionados com processo tumoral. No estudo, FN1, FGD3, TNN, ACAN, e ATP5F1B como as proteínas mais abundantes dentre as proteínas super expressas dos TMC. Assim, através de uma caracterização molecular de TMC e de análises de bioinformática, descrevemos proteínas-chave que podem ser ainda úteis na validação de biomarcadores para o câncer de mama tanto humano como canino.

Palavras-chave: células tumorais; estroma; transcriptômica; proteômica; oncologia comparativa; tumores mamários caninos; câncer de mama.

ABSTRACT

The present dissertation had two objectives: 1) to discuss the value of specific analyses in CMT using transcriptomics and proteomics for comparative oncology through a literature review; 2) to determine a comparative and quantitative analysis of proteins from CMT using the bottom-up proteomics approach. In chapter 1 we discussed the importance of using bitches with spontaneous mammary tumors as an animal model for HBC. The study of CMT can strongly contribute to the understanding of the disease course and contribute to the discovery of not only diagnostic but also prognostic and therapeutic biomarkers of breast cancer. The expression of tumor genes and proteins through the use of analyses such as transcriptomics and proteomics of tumor cells present in TMC have provided a wealth of data on gene expression, but the tumor stroma lacks studies. In chapter 2 we searched for candidate biomarkers of breast tumor status using a proteomic approach. We compared protein expression of canine mammary tumors (CMT) and normal mammary glands tissues (NT). Tissue samples (5 TMC and 5 NG) obtained from post-mortem female dogs from the Zoonosis Control Center (CCZ) were collected. The samples were divided into two portions. One portion was frozen and stored at -17◦C until used for proteomic analysis and the other portion followed standard biopsy procedures and was stored at room temperature. Benign mixed tumors (n=2), inflammatory carcinoma (n=1), complex adenomas (n=2), NT (n=5) were selected for shotgun proteomic approach. Peptide spectrum search was performed using Progenesis v4.2 which allowed the identification of an average of 945 proteins, 136 of which were overexpressed proteins in CMT. Most of these overexpressed proteins were involved in biological processes related to tumor process. In the study, FN1, FGD3, TNN, ACAN, and ATP5F1B as the most abundant proteins among TMC overexpressed proteins. Thus, through molecular characterization of CMT and bioinformatics analyses, we describe key proteins that may be further useful in validating biomarkers for both human and canine mammary cancer.

Keywords: tumor cells, stromal, transcriptomics, proteomics, comparative oncology, canine mammary tumors, breast cancer.

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LIST OF ABBREVIATIONS AND ACRONYMS

2DGE	Two-dimensional differential gel electrophoresis
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
CK8/18	Cytokeratin8/18
BSA	Bovine serum albumin
CAFs	Cancer associated fibroblasts
CAS	Cancer associated stromal
CCZ	Zoonosis Control Center
CMT	Canine Mammary Tumors
DCIS	Ductal carcinoma *in situ*
CSCs	Cancer stem cells
CTC	Circulating tumor cells
DDA	Data dependent acquisition
DFI	Disease free interval
ECM	Extracellular matrix
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen receptor
ERet	Endoplasmic reticulum
FFPE	Formalin fixed paraffin embedded
FMT	Feline mammary tumors
FrFr	Fresh frozen
GN	Glândulas mamárias normais
HBC	Human breast cancer
HER1	Human epidermal growth factor receptor 1
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
IC	Inflammatory carcinoma
IH	Immunohistochemistry
LC	Liquid chromatography
LCM	Laser capture microdissection
MALDI- TOF-MS	Laser assisted desorption time of flight/mass monitoring
mCA	Mammary carcinomas

miRNAs	Micro RNAs
MMPs	Matrix metalloproteinases
MS	Mass Spectometry
NCBI	National Center for Biotechnology Information
NT	Normal mammary glands tissues
OS	Overall survival
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNAseq	RNA sequencing
RT	Room temperature
RT qPCR	Real time qPCR
SEQ	Sequencing
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor beta
TIMPs	Tissue inhibitors of metalloproteinases
TMC	Tumores mamários caninos
TNF-β	Tumor necrosis factor beta
TNM	Tumor node metastasis
TP53	Tumor protein 53
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WB	Western immunoblotting
Wnt	Wingless type MMTV integration site
WHO	World Health Organization

SUMMARY

1 INITIAL CONSIDERATIONS

The spontaneous occurrence of cancer in the domestic dog is increasingly seen as a valuable model to further the understanding of cancer biology and potentially identify new therapeutic targets in dogs and human patients (GARDNER *et al.*, 2015; (Karlsson; Lindblad-toh, 2008). In particular, due to strong molecular properties and clinical similarities, canine mammary tumors (CMT) are considered an excellent model for human breast cancer (HBC) and are believed to overcome several of the limitations of xenografts or modified rodent tumor models (MARKANNEN, 2019).

Mammary tumors are the most common type in non-castrated female dogs and approximately half of these tumors are diagnosed as malignant, with the potential for metastasization, lymphogenous or hematogenous to other organs (Fazekas *et al.*, 2016). Other factors are related to a poor prognosis, such as a large tumor with skin ulcerations, histological grade, tumor stage, lymph node involvement, estrogen/progesterone receptors, p53 overexpression and mutations, and HER-2 overexpression are found in HBC and CMT (ABDELMEGEED 2018). Therefore, understanding the target genes and molecular mechanisms involved in the development and progression of CMT contributes to HBC and can collaborate in the development of new target therapies, discovery of potential drugs and biomarkers (Gray 2020).

Proteomics e transcriptomics of breast cancer have been successfully applied in order to discover biomarkers/protein signatures that are suitable for early diagnosis, tumor characterization, and subtyping; and prognosis and prediction of therapy outcomes (Gromov; Moreira; Gromova, 2014).

This dissertation aimed in chapter 1 to discuss the value of specific analyses in CMT using transcriptomics and proteomics for comparative oncology; while in chapter 2 the objective was to perform a comparative and quantitative analysis of proteins from CMT using bottom-up proteomics.

2 CHAPTER 1: THE VALUE OF SPECIFIC ANALYSES OF CANINE MAMMARY TUMOR CELLS AND THEIR STROMAL USING TRANSCRIPTOMICS AND PROTEOMICS FOR COMPARATIVE ONCOLOGY.

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O VALOR DE ANÁLISES ESPECÍFICAS DE CÉLULAS TUMORAIS MAMÁRIAS CANINAS E SEU ESTROMA USANDO TRANSCRIPTÔMICA E PROTEÔMICA PARA A ONCOLOGIA COMPARATIVA.

RESUMO

O câncer de mama é uma malignidade agressiva que frequentemente causa metástase e o tratamento individualizado fornece melhores resultados terapêuticos aos pacientes acometidos por esta enfermidade. O desenvolvimento de um modelo animal que apresenta tumores mamários espontâneos pode ajudar na compreensão da tumorigênese, progressão e mecanismos de metástases do câncer de mama. O estudo dos TMC pode contribuir para a descoberta de biomarcadores não só de diagnóstico, como também de biomarcadores prognósticos e terapêuticos. Este último permite aos clínicos estratificar os pacientes, sejam eles caninos ou humanos, para prever a resposta ao tratamento e o resultado. Esta revisão aborda a importância do estudo dos TMC caninos na oncologia comparativa e os biomarcadores moleculares identificados em amostras tumorais que têm sido explorados até à data. A expressão de genes e proteínas de tumores independentemente da morfologia celular ou das características dos tecidos, está sendo cada vez mais utilizada tanto no campo humano como veterinário para ajudar no diagnóstico e prognóstico do câncer. Nos últimos anos, análises como transcriptômica e proteômica de células tumorais presentes em TMC forneceram uma riqueza de dados sobre a expressão genética, porém o estroma tumoral carece de estudos. O estroma tumoral influencia diretamente a iniciação e o desenvolvimento do câncer de mama humano e o mesmo acontece em cães. Finalmente, propomos uma investigação mais profunda do estroma tumoral no diagnóstico dos tumores mamários caninos para estabelecer a relação entre os componentes do estroma e as células neoplásicas.

Palavras-chave: células tumorais; estroma; transcriptômica; proteômica; oncologia comparativa; tumores mamários caninos; câncer de mama.

THE VALUE OF SPECIFIC ANALYSES OF CANINE MAMMARY TUMOR CELLS AND THEIR STROMAL USING TRANSCRIPTOMICS AND PROTEOMICS FOR COMPARATIVE ONCOLOGY.

ABSTRACT

Breast cancer is an aggressive malignancy that often causes metastasis, and an individual treatment can provide better therapeutic outcomes for patients affected by this disease. The development of an animal model that presents spontaneous mammary tumors may help in the understanding of tumorigenesis, progression, and metastasis mechanisms of breast cancer. The study of canine mammary tumors (CMT) may contribute to the discovery of not only diagnostic, but also prognostic and therapeutic biomarkers. The latter allows clinicians to stratify patients, whether canine or human, to predict treatment response and outcome. This review addresses the importance of studying CMT in comparative oncology and the molecular biomarkers identified in tumor samples that have been explored to date. The expression of tumor genes and proteins regardless of cell morphology or tissue characteristics is increasingly being used in both human and veterinary fields to aid in cancer diagnosis and prognosis. In recent years, analyses such as transcriptomics and proteomics of tumor cells present in CMT have provided a wealth of data on gene expression, however the tumor stroma has been lacking of studies. The tumor stroma directly influences the initiation and development of human breast cancer (HBC) and the same is true in dogs. Finally, we propose further investigation of tumor stroma in CMT diagnosis to establish the relationship between stromal components and neoplastic cells.

Keywords: tumor cells, stromal, transcriptomics, proteomics, comparative oncology, canine mammary tumors, breast cancer.

2.1 Introduction

Female breast cancer constitutes a large part of the total neoplasia affecting women and it is the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%), according to GLOBOCAN 2020 (Sung *et al.*, 2021). The early diagnostic possibility of more survival rate after the therapy of patients advances the technique of surgery and appropriate treatment (Gray *et al.*, 2020). It is also fundamental to establish an animal model that developed breast cancer in a manner spontaneous with translational relevance to women (ANTUOFERMO *et al.*, 2007).

The studies about cancer in dogs to answer questions about cancer therapies in humans are not novel, they were reported since the 1960s and have many efforts underway in the United States, Europe, and other countries that suggest a strong interest in comparative oncology (PAOLONI; KHANNA, 2008). The occurrence of spontaneous mammary tumors in some species of animals, mainly companion species (pet), has been highlighted as a model for human cancer over the last four decades in the understanding of tumorigenesis, progression, and metastasis. Thus, it provides an improvement in diagnostic and treatment approaches for humans and animals (VISAN *et al.*, 2016).

Domestication provides dogs living in the same human environment and they are exposed to many of the same carcinogens. Factors like obesity, diet, breeding fixation and consanguineous crosses in some breeds of dogs promote the incidence to develop certain cancer types, facilitating the identification of risk alleles related to cancer (Gray *et al.*, 2020; Markkanen, 2019). Because dogs have a shorter life than humans the progression of cancer is faster, therefore, it is possible to collect data and conclusion of the case study in a brief time (Antuofermo *et al.*, 2007). Furthermore, the advancing age, the hormonal etiology, and the identical course of the disease allied to dogs receive a better health assistance risk if compared to rodent models (ABDELMEGEED; MOHAMMED, 2018; MARKKANEN 2019, GRAY *et al.*, 2020; SCHIFFMAN; BREEN, 2015).

A study published in 2006 analyzed the gaps in search areas about breast cancer. Several important scientists in this area contributed and the gaps were targeted by other researchers and funding bodies with recommendations for future research. They highlight some points like the initiation of breast cancer, early progression regulators, prevention, disease marker, therapies, and targets. Besides, some problems are related to an appropriate animal

model and a lack of clinical samples that could be solved by increasing the use of spontaneous animal models with translational relevance (Thompson *et al.*, 2006).

Canine mammary tumors show similarities in genetic and protein structure as well as expression level and pattern, along with signaling pathways common to HBC. In this connection, the use of tools such as transcriptomics and proteomics provides a greater understanding of the molecular underpinnings of tumor cells and their stroma in both the canines and humans. This holds enormous potential for interesting discoveries and also to identify conserved aspects that are probable causes of the disease for both species.

2.1.1 What can we learn from studying canine mammary tumors for human breast cancer?

Human breast cancer is the most common cancer among women and it is a heterogeneous disease with complex molecular processes (Momenimovahed; Salehiniya, 2019; Stingl; Caldas, 2007). Coincidentally, mammary cancer is the most diagnosed cancer in female dogs and many authors claim that CMT are suitable models for the study of HBC because of histological, clinicopathological, and molecular homologies with the human disease (Fazekas *et al.*, 2016; Gray *et al.*, 2020; Liu *et al.*, 2014; Queiroga *et al.*, 2011; Visan *et al.*, 2016). Moreover, two characteristics make CMT a suitable model for human research. First, tumor initiation and progression occur spontaneously in canines, by disputing suggested limitations of genetically engineered mice being more authentic models to faithfully mimic the human disease (Wagner, 2004). Second, dogs share a common living environment with humans, unlike any laboratory animal. Therefore, this represents similar interactions that exist between genetics and environmental risk factors associated with both human and CMT. Looking at these two singularities, the spontaneous occurrence of tumors, as well as the fact that canines usually share the same environment with humans, the study of CMT, represents a remarkable opportunity to improve our knowledge of HBC, allowing us to assess everything from physiology to treatment (Gardner; Fenger; London, 2016; Gray *et al.*, 2020; Karlsson; Lindblad-toh, 2008; Liu *et al.*, 2014; Markkanen, 2019; Schiffman; Breen, 2015). However, comparative oncology as a pathway to accelerate drug development using dogs is still underutilized.

Studies have already demonstrated many characteristics of common CMT with HBC, including clinical and molecular similarities (MARCHESI *et al.*, 2010; GRAY *et al.*, 2020; ZHAO *et al.*, 2021). Despite the differences between canine and human lifespans, the

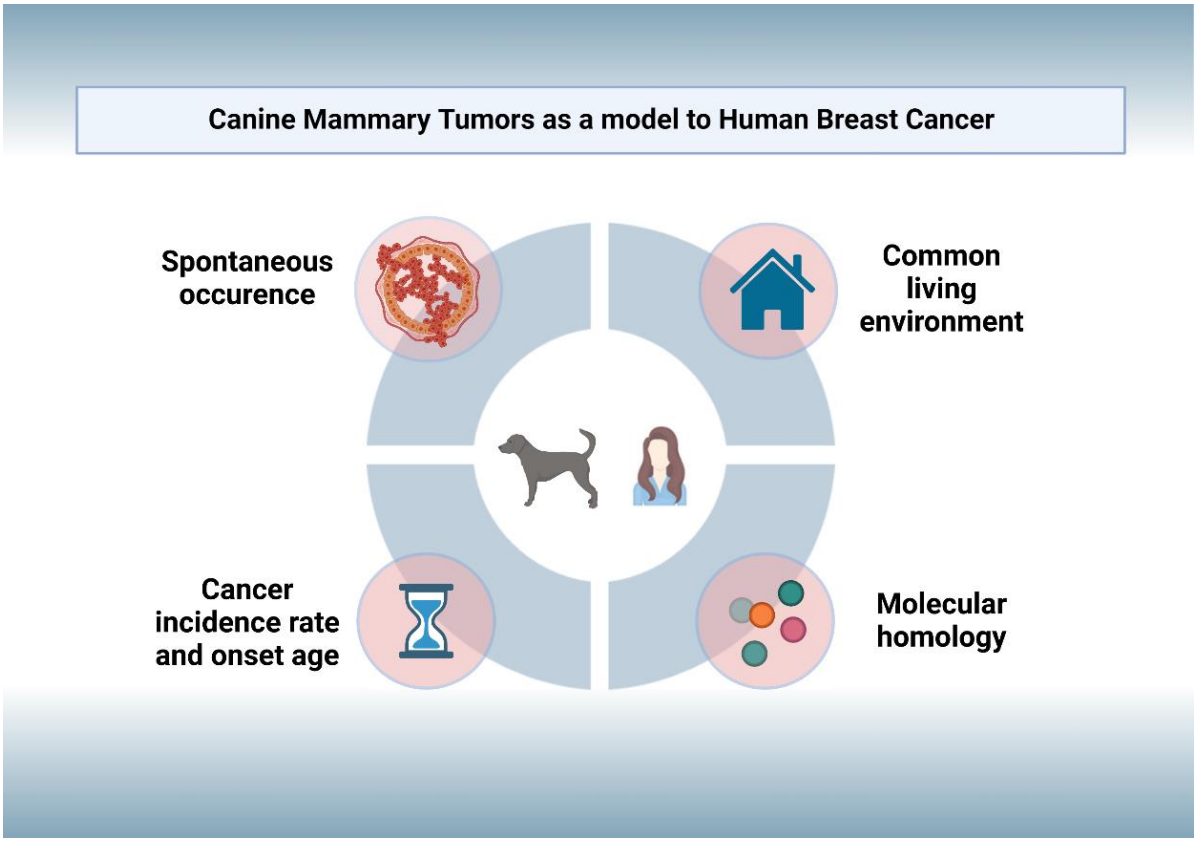
average onset of mammary cancer in canines (at 6 years) and humans (at 40 years) occur at equivalent stages of life for these two species. Additionally, the peak incidence of CMT coincides in canines and women when the ages of the species are compensated (Queiroga *et al.*, 2011).

In the mammary gland of humans and canines, the epithelium is formed by lobules and alveoli, divided by connective tissue. In addition, the secretory epithelium of both species is composed of two main cellular lineages: luminal cells that surround a central lumen and a layer of surrounding myoepithelial cells that are located in a basal position adjacent to the basement membrane (VISVADER; STINGL, 2014; SLEECKX *et al.*, 2011). Myoepithelial cells in CMT proliferate more frequently than in HBC, occurring in more than 20% of all CMT cases but in less than 0.1% of HBC cases. Because myoepithelial cell proliferation in HBC is rarely found, insufficient attention has been devoted to studying these cells, limiting data exchange and applications in comparative oncology (LIU *et al.*, 2014; SLEECKX *et al.*, 2011). Myoepithelial cells in CMT proliferate more commonly than in HBC, occurring in more than 20% of all CMT cases, but in less than 0.1% of HBC cases. Because myoepithelial cell proliferation in HBC is rarely found, not sufficient attention has been devoted to studying these cells, limiting data exchange and applications in comparative oncology (Liu *et al.*, 2014).

Another relevant aspect is the great similarity of molecular and histological heterogeneity between CMT and HBC (Valdivia *et al.*, 2021). In addition, the similarity between human and canine genome organizations is more than between human and mouse, which is evidence that the dog may be a better model for human cancer studies (Park *et al.*, 2016). In this context, the study of CMT provides a valuable perspective for the identification of cancer-associated genes and molecular players in breast cancer. Canines have a shorter life expectancy compared to humans, which allows researchers to study the development of cancer in relatively short periods (Gray *et al.*, 2020) and canine experiments certainly provide a plethora of tissue samples and potential inclusion in clinical trials (Abdelmegeed; Mohammed, 2018). CMT research brings valuable information not only about the biology and progression of tumor cells but also about the synergy between cancer cells and their stromal (Markkanen, 2019; Paoloni; Khanna, 2008). The stromal is a heterogeneous arrangement of non-cancer cells (fibroblasts, vascular cells, immune cells, adipocytes, and others) and the extracellular matrix, which surrounds cancer cells and supports tumor initiation and progression (Pöschel *et al.*, 2021). There is increasing evidence that this arrangement of non-cancer cells promotes events that favor disease progressions such as tumor angiogenesis, proliferation, invasion, and metastasis, and even develops mechanisms of therapeutic resistance (Bussard *et al.*, 2016).

In recent decades, new insights about molecular mechanisms associated with HBC cells have provided major advances in the development of better markers for prognosis and treatments for the disease (Mueller; Fusenig, 2004). However, the stromal associated with cancer is not completely understood yet because of its complexity and biological role involving antagonistic tumor-promoting and inhibiting pathways (Rowley, 2014). Studies about the stromal in CMT and its influence on tumor progression are poorly understood when compared to HBC. Knowledge of these parameters is essential for strengthening the canine animal model concept, as well as for successful diagnosis and treatment for both canines and humans (Markkanen, 2019).

Figure 1 - Schematic presentation of how CMT can be a model to HBC in comparative oncology field.



2.1.2 Molecular mechanisms and biomarkers in CMT

Understanding the molecular mechanisms of the disease supports efforts to discover biomarkers for early cancer diagnosis and safety evaluations in prognosis, metastasis prediction, and monitoring clinical response to a given intervention (Atkinson *et al.*, 2001). Biomarkers also have applications for the development of new target therapies and potential drugs for both human and canine species. Unfortunately, the identification of biomarkers and the assessment of molecular signatures are still restricted to the field of fundamental research, with limited applications in veterinary medicine practice (Gray *et al.*, 2020).

Molecular profiling of tumor tissue is a valid strategy to understand how tumor development and progression work and many tissue biomarkers identified in CMT have similar characteristics to HBC. Pathways and gene expression related to tumor malignancy in CMT promote cell proliferation (Ki-67 and PCNA) and apoptosis (p53) respectively, and such factors are considered biomarkers for the human model as well. Ki-67 is the most sensitive marker of cell proliferation and is found in all stages of the cell cycle. Overexpression of Ki-67 and PCNA corresponds to poor prognosis and shorter OS in both canines and humans. Mutations in the p53 gene are commonly found in human and canine mammary tumors and p53 overexpression is associated with higher tumor aggressiveness (Abdelmegeed; Mohammed, 2018; Carvalho *et al.*, 2016; Muto *et al.*, 2000; Peña *et al.*, 2003).

Proteins involved in cell adhesion such as E-cadherin (epithelial-cadherin) and MUC 1 indicate the metastatic potential of CMT. During tumor growth, inflammatory cells secrete pro-inflammatory mediators like COX-2 and there are changes in the expression of angiogenic molecules such as VEGF, EGFR (HER1), and HER-2. Overexpression of HER-2 is unequivocally related to tumor growth, survival, and differentiation of cells and it is equally expressed in both HBC and CMT. In humans, HER2 expression is evaluated along with ER and PR expression for the staging of HBC (Kaszak *et al.*, 2018). As well known, ovarian hormones, especially estrogen, act as inducers of mammary neoplasms in both humans and canines (Sorenmo *et al.*, 2019). In addition, pseudopregnancy and contraceptive use contribute to CMT formation (Kaszak *et al.*, 2018) and epidemiological data from countries where dogs are spayed at early ages show a significant reduction in the incidence of mammary tumors in pre-pubertal spayed dogs when compared to intact or dogs spayed at older ages (Kristiansen *et al.*, 2016; Queiroga *et al.*, 2011). The interaction of estrogen with its receptors and estrogen effects on target tissues are probably the same in dogs and humans, but the differences in the reproductive cycles of bitches and women may be considered problematic for comparative

oncology. However, dog estrus cycles allow us to study cancer in an estrogen-rich or estrogen-deprived natural environment due to the extended period of each phase (Sorenmo *et al.*, 2019).

BRCA1 and BRCA2 are tumor suppressor genes and play critical roles as DNA damage sensors and inducers of the DNA repair response. Germline mutations in BRCA1 and BRCA2 are associated with an enhanced risk of cancer predisposition in humans and 5% to 10% of human breast cancer cases are considered hereditary (Schiffman; Breen, 2015). In contrast to studies conducted in humans, there is no confirmed evidence that CMT has any hereditary pattern, although some breeds have a predisposition for the development of mammary tumors (Rivera *et al.*, 2009). Despite the relevance of BRCA1 and BRCA2 mutations for the prognosis of HBC, studies revealed no association between CMT and genomic polymorphisms of BRCA1 and BRCA2. Malignant CMT has increased cytoplasmic and reduced nuclear expression of BRCA1 protein, although changes in location and expression are not significantly associated with disease-free interval (DFI) and OS (Klopfleisch *et al.*, 2011; Nieto *et al.*, 2003). Given the anatomical similarities and coincident molecular events, the dog is indeed a reliable model for the study of HBC. In the near future, significant advances in comparative oncology research will provide more opportunities to leverage this natural model, especially by investigating the potential agents involved in both CMT and HBC (Table 1).

Table 1 - Summary of relevant molecular markers in CMT, their functional attributes related to cancer pathobiology, value as a biomarker, and associations with DFI and OS. Main links with HBC and feline mammary tumors are also listed.

CANINE						HUMAN	FELINE
Molecular factors	**Methods**	**Functional attributes / cancer pathobiology**	**Biomarker value for CMT**	**DFI**	**OS**		
Marker of proliferation Ki-67* E2RJX3 (E2RJX3_CANLF) *	IH (Brunetti et al., 2021)	Cell proliferation (Brunetti et al., 2016)	High expression related to poor clinical, large tumor size, inflammation and ulceration, metastases to lymph nodes (Rodrigues et al., 2016; Carvalho et al., 2016).	High Ki-67 is related to low DFI (Zuccari et al., 2008).	High Ki-67 is related to shorter OS (Carvalho, 2016).	High Ki-67 is related to poor prognosis, but shows good response to chemotherapy (Emi et al., 2016).	High Ki-67is related to increase of cell proliferation and activity (Campos et al., 2016).
Proliferating cell nuclear antigen - PCNA* (E2R0D6_CANLF) *	IH (Carvalho et al., 2016)	Marker of the proliferation index. DNA replication, related to DNA excision repair, cell cycle control, chromatin assembly, and RNA transcription (Jurikova et al., 2016).	Related to tumor size, histological type, differentiation grade, nuclear grade, mitotic index, histological grade of malignancy, and lymph node metastasis (Carvalho et al., 2016).	No association (Zuccari et al., 1998)	High PCNA is associated to shorter OS (Carvalho et al., 2016)	PCNA is evaluated together with other HBC biomarkers, such ER, PR, Ki-67 or HER-2 (Juríková et al., 2016)	Not correlated with nuclear grade in cats, (Deveci et al., 2021)
Cellular tumor antigen p53*	IH (Bulka et al.,	Cell cycle control, apoptosis,	Prognostic market. Related to higher mitotic count, invasive growth, and	p53 high expression is related to poor	p53 high expression is related to poor	High expression of p53 is related to worse prognosis and shorter	High p53 expression is related to

Q29537 (P53_CANLF) *	2016; Brunetti et al., 2021).	suppressor of tumor growth (Sorenmo, 2003).	necrosis (Dolka et al., 2016; Brunetti et al., 2021).	DFI (Klopfleisch and Gruber, 2009a).	OS (Klopfleisch and Gruber, 2009a).	OS (Bertheau et al., 2013).	malignancy (Nakano et al., 2006).
Cadherin-1 – E-cadherin* F1PAA9 (CADH1_CANLF) *	IH (Matos et al., 2006).	Cellular adhesion (Matos et al., 2006).	Prognostic market, high levels are related to aggressiveness and a poor prognosis (Matos et al., 2006).	E-cadherin low expression is related to short DFI (Gama et al., 2008; Matos et al., 2006).	E-cadherin low expression is related to shorter OS (Gama et al., 2008; Matos et al., 2006).	Down expression is related to histological grade, tumor size, lymph node status, worse prognosis (Gama et al., 2008)	Decrease expression is related to malignant tumors and metastases (Zapulli et al., 2012).
Mucin 1, cell surface – MUC-1* F1Q3G6 (F1Q3G6_CANLF) *	IH (Campos et al., 2015; Manuali et al., 2012). WB and IH (Manuali et al., 2012).	Cell proliferation, apoptosis, adhesion and invasion (Manuali et al., 2012).	High Mucin 1 is related to poor clinical stage and bad prognosis (Manuali et al., 2012).			High MUC-1 is related to angiogenesis, progression, and metastasis (Rachagani et al., 2009).	
Vascular endothelial growth factor A - VEGF* Q9MYV3 (VEGFA_CANLF) *	IH (Queiroga et al., 2011).	Angiogenic factors (Queiroga et al., 2011).	High VEGF is found in malignant tumors and benign tumors (Queiroga et al., 2011).	No association (Santos et al 2013).	No association (Santos et al 2013).	High levels of VEGF are found in HBC (Verrax et al., 2011)	High levels are related to clinical outcome (Millanta et al., 2002)

92kDa gelatinase – MMP-9* F1PYF5 (F1PYF5 CANLF) *	IH and WB (Chen et al., 2019).	Regulates pathological remodeling processes that involve inflammation (Mondal et al., 2020).	High MMP-9 is related to malignant tumor (Chen et al. 2019).	High expression of MMP-9 is related to poor DFI (Santos, 2013).	High level is related to poor OS (Santos, 2013).	The expression of MMP-9 is upregulated in different types of cancer, including HBC (Liu et al., 2012)	Related to progression and metastatic carcinomas (Akkoc et al., 2012).
Cyclooxygenase-2 – COX-2* Q8SPQ9 (Q8SPQ9_CANLF)	IH (Lavalle et al., 2009)	Tumor growth, prognosis and tumorigenesis (Carvalho et al., 2017).	Increased expression is related to increasing malignancy of CMT (Klopfleisch et al., 2011).	High levels are related to poor DFI (Queiroga et al., 2010).	High levels are related to poor OS (Queiroga et al., 2010; Zappulli et al, 2015).	High COX-2 is related to development and progression of the malignancy (Singh et al., 2007).	High COX-2 related to malignancy (Millanta et al., 2006).
Epidermal growth factor receptor (EGFR, HER-1 or c-erbB1) **Q9TTY2 (FER_CANLF) ***	IH (Carvalho et al., 2013)	Angiogenesis, cancer cell migration and invasion (Kandefer-Gola et al., 2016).	Increased EGFR is related to high malignancy grade, poor clinical stage and large tumor size (Carvalho et al., 2013).	Positive EGFR expression leads to short DFI (Gama et al., 2009).	Presence of EGFR trending toward a shorted OS (Gama et al., 2009).	Half of cases of triple-negative HBC and inflammatory HBC overexpress EGFR (Masuda et al., 2012).	Overexpressed in feline mammary carcinomas (Wiese et al., 2013).
Receptor tyrosine-protein kinase (erbB-2 – HER-2) * O18735 (ERBB2_CANLF)	IH, WB, MS, qRT PCR (Burrai et al., 2015)	Regulate tumor growth, survival and differentiation (Campos et al., 2015).	Related to carcinogenesis (Campos, 2015). Positive correlation with expression and tumor mitotic index, high histological grade and size (Dutra et al., 2004).	High HER2 is related to DFI after surgery less than 6 months (Martín de las Mulas, 2005).	Increase OS in dogs with malignant CMT overexpressing HER2 (Shinoda, 2014).	High HER-2 is strongly related to decreased OS (De Pedro et al., 2015).	Related to aggressive clinicopathological features, with HER2-positive being the most frequent subtype (Gameiro et al., 2021).

Estrogen receptor - ER* F6V0I8 (F6V0I8_CANLF) *	IH (Millanta et al., 2005; Brunetti et al., 2021).	Promotes the transcription of proliferative genes and suppresses apoptotic genes (Yue et al., 2013).	ER decreased in malignant CMT (Millanta et a., 2005; Brunetti et al., 2021).	High ER is related to shorter DFI. (De Las Mulas et al., 2005).	Strong correlation with OS (Brunetti et al., 2021).	ER and PR levels are routinely evaluated to assess response to endocrine therapy (Hammond et al.,2010).	ER and PR levels in invasive carcinomas are not correlate with other histological parameters or OS (Hughes et al., 2012; Millanta et al., 2005).
Progesterone receptor – PR* (Q9GLW0 (PRGR_CANLF)	IH (Mainenti et al., 2014).	Play major roles in the normal development of the mammary glands. PR implicated in tumor growth (Spoerri et al., 2015).	PR expression is progressively decreased in hyperplastic/ dysplastic, benign, and malignant canine mammary lesions. (Milanta et al., 2005).	PR expression is related to higher DFI than those lacking PR expression (Chang et al., 2009).	PR expression is related to higher OS rates than those lacking PR expression (Chang et al., 2009).	Expression of PR is more frequent in well differentiated tumors than in poorly differentiated ones. Related to progression from ductal carcinoma in situ to invasive carcinoma (Cenciarini and Proietti, 2019).	PR expression is increased from healthy and dysplastic to neoplastic lesions (Millanta et al., 2005).
Breast cancer type 1* Q95153 (BRCA1_CANLF) * BRCA1**	IH (Nieto et al., 2003); SEQ (Barrios et al., 2017); RTqPCR (Klopfleisch and Gruber et al., 2009b)	Regulation of the cell cycle (Nieto et al., 2003)	Low BRCA1 expression is related to high proliferation a poor prognosis, (Nieto et al 2003). Gene expression is related to malignancy (Klopfleisch and Gruber et al., 2009b).	Expression are not associated (Nieto et al., 2003).	Expression are not associated (Nieto et al., 2003).	Reduced BRCA1 mRNA levels are related to nonsense mutations and lead to an increased resistance to apoptosis (Klopfleisch and Gruber et al., 2009b).	Spontaneous animal model for studying novel therapeutic approaches against HBC (Wiese et al., 2013).

Breast cancer 2* (IOIV85_CANFL) **BRCA2****	RTqPCR (Yoshikawa et al., 2015; Klopfleisch and Gruber et al., 2009b); SEQ (Yoshikawa et al., 2015)	Tumor suppressor genes play important roles in DNA repair and apoptosis (Yoshikawa et al., 2015)	BRCA2 is related to homologous recombination repair via its interaction with RAD51 recombinase, and this function suppresses tumorigenesis (Yoshikawa et al., 2015).	Expression are not associated (Hsu et al., 2010)	Expression are not associated (Hsu et al., 2010)	Mutation is related to a more heterogeneous group of tumors with a higher prevalence in luminal-A subtype HBC (Bane et al., 2009)	BRCA 2 sequencing is not identify mutations similar to those described in human (Wiese et al., 2013).

* Protein description and Uniprot accession code; ** Oficial gene symbol (NCBI); IH-Immunohistochemistry; WB- Western immunoblotting; MS- Mass Spectometry; SEQ- Sequencing.

2.1.3 Comparative overview of spontaneous CMT and HBC

Most cases of spontaneous CMT consist of circumscribed nodules of different sizes, textures, locations (60% located in caudal glands), and molecular subtypes (luminal A, luminal B, HER2-enriched, and basal-like) (Queiroga *et al.*, 2011). CMT differ in regard to histopathological classification and present complex morphology related to epithelial, mesenchymal, or mixed type cells (CASSALI *et al.*, 2014; VISAN *et al.*, 2016). CMT can be associated with ulcerations of the skin near the mammary gland and this feature relates to poor prognosis and OS. Additional relevant criteria associated with poor prognosis and OS of CMT cases are tumor size (>3 cm), tumor stage, presence of significant nuclear and cellular pleomorphism, mitotic index, and lymph node involvement (Goldschmidt *et al.*, 2011; Nunes *et al.*, 2018). Canines may develop multiple tumors in a single mammary gland or tumors in more than one gland at the same time. In the case of multiple tumors, the tumor with the worst prognosis is the leading reference for the best treatment for the patient (Cassali *et al.*, 2014). Approximately half of spontaneous CMT are diagnosed as malignant and all of these malignant tumors have the potential to metastasize in the lymphatic or hematogenic pathways associated with lower OS (Fazekas *et al.*, 2016; Nunes *et al.*, 2018). Malignant mammary tumors may recur after surgical intervention and metastasize to distant organs in both canine and human species (Visan *et al.*, 2016).

2.1.3.1 Canine simple mammary carcinomas

Generally, the spontaneous canine mammary tumors are defined as either simple or complex carcinomas. While canine simple mammary carcinomas (mCA) are valid models for spontaneous HBC, canine complex mammary carcinomas receive much less attention because they present proliferation of myoepithelial cells, and this type of neoplasia rarely occurs in HBC. Besides, extensive genomic aberrations are frequent in canine simple mCA but uncommon in canine complex carcinomas, and many of these aberrations truly resemble human disease (Liu *et al.*, 2014). Canine simple mCA are composed of only one cell type, resembling both luminal epithelial cells and myoepithelial cells. This type of carcinoma presents neoplastic cells that infiltrate the surrounding tissue, where they induce a strong stromal response that may reach lymphatic vessels, causing metastasis (GOLDSCHMIDT *et al.*, 2011). At the molecular level, canine simple mCA replicates the genomic alterations found in HBC such as amplification and/or overexpression of oncogenes, deletion and/or low expression of tumor suppressors, and mutation of BRCA1. Canine simple mCA and HBC also share similar altered

molecular pathways such as those related to cell adhesion, Wnt signaling, PI3K signaling, and DNA repair (Liu *et al.*, 2014). In fact, all these homologous features make spontaneous canine simple mCA a valuable source of research for HBC.

Approximately, half of malignant CMT (including canine simple mCA) have reduced prognosis and potential for metastasis. Canine simple mCA rarely presents bone metastasis but most commonly has metastasis in the thoracic region (lymph node and lung). This canine neoplasia can be as lethal and aggressive as HBC, and it is essential to identify groups at risk of recurrence and mortality risk (Queiroga *et al.*, 2011). However, the characterization of molecular mechanisms associated with reduced prognosis and metastasis in HBC and canine simple mCA is still largely unknown as well as why some carcinomas metastasize and others do not (Gray *et al.*, 2020; Metastatic canine mammary carcinomas can be identified by a gene expression profile that partly overlaps with human breast cancer profilesKlopfleisch, Robert *et al.*, 2010).

2.1.3.2 Inflammatory mammary carcinoma

Inflammatory carcinoma (IC) is a rare and aggressive subtype of breast cancer affecting both canines and humans. IC has a high metastatic rate, with an incidence of nearly 7% of all CMT and 5% of all HBC cases (Giordano; Hortobagyi, 2003; Sleeckx *et al.*, 2011). The low incidence of IC in spontaneous CMT and the difficulties in its diagnosis explain the limited number of studies published about canine IC (ALENZA MD; TABANERA E, 2001; PEÑA *et al.*, 2003). Canines and felines are the only non-human species that presenting IC (Peña *et al.*, 2003; Raposo *et al.*, 2016) and authors have suggested that canine IC is a valid model for humans (ALENZA M & TABANERA E, 2001; PEÑA *et al.*, 2003; RAPOSO *et al.*, 2016; QUEIROGA *et al.*, 2005). Canine and human IC are considered the most malignant type of breast cancer and present distinct genetic, biological, and clinical features when compared to other types of mammary neoplasms. The clinical features are similar in canine and human inflammatory breast cancer, and the disease has two forms, one before surgery and one occurring after the surgery, with aggressive behavior. Even after the surgery, IC can take on a unique feature in both species, which is embolization of the dermal lymphatic vessels due to blockage of the lymphatic drainage, so-called “occult” IC (ALENZA MD; TABANERA E, 2001; PEÑA *et al.*, 2003). The expression of molecular agents related to inflammation and malignancy, such as COX-2 and VEGF, have prognostic value for both canine and human mammary IC, and genes involved in tumor microenvironment biology, such as TRIB1, SNCG, and CSF1R, are differentially expressed in IC and other malignant canine mammary tumors.

These molecular aspects explain the aggressive behavior of canine IC tumors and boost further research aimed at comparing it with human inflammatory carcinoma (Raposo *et al.*, 2016).

2.1.3.3. Ductal carcinoma in situ

Ductal carcinoma in situ (DCIS) is characterized by increased proliferation of malignant neoplastic cells limited to the ductal area without invasion outside the breast ductal system. In humans, DCIS is a precursor of invasive breast cancer, but the mechanisms driving the progression of DCIS to invasive breast cancer are unclear (Doebar *et al.*, 2016). Visual, pathological, molecular, clinical, and imaging characteristics of DCIS are similar in dogs and humans (Abdelmegeed; Mohammed, 2018). Pre-invasive lesions such as atypical ductal hyperplasia and DCIS can develop before cancer cells enter the adjacent canine mammary tissue, just as in humans (Gilbertson *et al.*, 1983). Several molecular markers of canine DCSI are similar to those detected in human ductal breast cancer, including estrogen and progesterone receptors, and Ki-67 and HER2 expressions as well (Antuofermo *et al.*, 2007).

Researchers have used methods in transcriptomics for the identification of potential targets and pathways related to the progression of canine DCIS to invasive cancer. It is believed that myoepithelial cells and the basement membrane that circumscribes DCIS are lost during tumoral progression, allowing tumor cells to invade the stroma microenvironment and the adjacent tissue. Studies report that myoepithelial cells markers such as MMP11, COL1A2, COL3A1, COL8A1, S100A2, and FN1 are overexpressed in canine DCIS. In addition, DCIS and invasive cancer have different expressions of FZD2, SFRP2, STK31, and LALBA, which are the core Wnt pathway components (Mohammed *et al.*, 2020). Given this scenario, there is an urgent need for the identification of molecular markers that can stratify DCIS lesions and prevent DCIS from becoming invasive. The similarities between canine DCIS and the human condition justify the dog as a valid model for the human disease and for translational comparative oncology. However, detection of canine DCIS at early ages is challenging because DCIS is commonly asymptomatic in both dogs and humans.

2.1.4 Feline mammary tumors

Over the past four decades, the occurrence of spontaneous mammary tumors in non-human species, mainly domestic canines and felines, has helped our understanding of the factors involved in HBC tumorigenesis, progression, and metastasis. However, domestic

felines have not been used as a model for human cancer to the same degree as canines (VISAN *et al.*, 2016; CANNON, 2015) despite the similarities between feline mammary tumors (FMT) and HBC. One problem for defining FMT as a model for humans is the scarcity of feline samples and the heterogeneity of follow-up studies, mainly because cat owners usually do not adhere to long-term cancer treatments (Abdelmegeed; Mohammed, 2018).

Feline mammary tumors are the third most common neoplasm in female felines. Frequently, felines receive a late diagnosis, presenting tumors with ulceration mainly associated with extensive tumoral necrosis (Zappulli *et al.*, 2005). FMT are more common in non-spayed felines because mammary tumors are associated with estrogen and progesterone actions, as well as with oral contraceptive administration (Ferreira *et al.*, 2019; Gameiro; Urbano; Ferreira, 2021). At least 80% of all FMT are malignant and defined as carcinomas with an aggressive potential of lymph node invasion and metastasis to the thoracic region or distant regions (De Campos *et al.*, 2016). Mammary tumors arise in middle-aged to old feline females (10 to 12 years old) and have anatomical, histopathological, genetic, and molecular features that coincide with HBC, highlighting their importance for comparative oncology (Zappulli *et al.*, 2005).

Human breast cancer is characterized according to molecular subtypes: luminal a, luminal b, HER2+, and triple-negative (normal type and basal type). In recent years, FMT has emerged as a valuable model for HBC, especially for clinical trials of HER2+ and triple-negative breast carcinoma therapies (Gameiro; Urbano; Ferreira, 2021). Mammary carcinomas with the HER2+ subtype are characterized by HER2 overexpression and the absence of receptors for estrogen and/or progesterone. On the other hand, tumors defined as triple-negative do not present expression for ER, PR, and HER2+ (Nascimento; Ferreira, 2021). The expression of ER and PR receptors, HER2+, VEGF, and COX enzymes, as well as Ki-67 proliferative index, have been studied in feline mammary neoplasms and may represent a therapeutic option that could influence OS (De Campos *et al.*, 2016). In addition, FMT altered the expression of cancer-related genes such as TP53, YBX1, CCND1, PTBP1, FUS, c-MYC, and PKM2. These genes are well known from HBC, but little is known about the molecular mechanisms originating from these genes in FMT. Transcriptomic analyses have revealed several associations of these genes with clinicopathological parameters of FMT, and also evidence that these genes may indirectly influence other genes establishing a molecular cancer network that is important to analyze as a whole, especially to strengthen FMT as models for HBC (Ferreira *et al.*, 2019).

The screening process and identification of early biomarkers are important to improve the survival rate of felines with mammary carcinomas (Zheng *et al.*, 2020). Proteomic approaches have been widely used in human cancer research but rarely in comparative oncology. Immunohistochemistry, instead, is more commonly used for analysis of protein expression in veterinary oncology (Kycko; Reichert, 2014). In fact, immunochemistry has been used to validate gene and/or protein expression in FMT over the years (Cherrington *et al.*, 2012; Dagher *et al.*, 2020; Levels *et al.*, 2020; Millanta *et al.*, 2005; Ressel *et al.*, 2009; Santos *et al.*, 2013). However, feline mammary tumors still lack molecular characterization based on high-throughput technologies, not only of the bulk tumor tissue but also for the tumor stromal.

2.1.5 Similarities between canine and human mammary tumor cells

In multicellular organisms, cell birth and cell death are kept in balance for the maintenance of normal biological mechanisms and then, cell division, proliferation, and differentiation are strictly controlled, and derangement of this balance leads to disordered cell proliferation and possibly tumor formation (Argyle; Nasir, 2003). Most cancers originate from mutationally corrupted epithelial cells which generate cancer cells that proliferate, progress, and enter the surrounding microenvironment. These epithelial tumor cells are not self-sufficient and, therefore, they do need to the components of their microenvironment for maintenance and growth (Argyle; Nasir, 2003; Hanahan; Coussens, 2012).

In recent decades, scientists have been studying the roles of oncogenes and tumor suppressor genes, as well as new principles, signaling pathways, and molecules that define cancer cells (Hanahan; Coussens, 2012). Human breast cancer is derived from the gradual accumulation of genetic changes, and most of these changes are linked to two types of mutational genes: one is the proto-oncogenes, which are responsible for stimulating the growth of normal cells, and the other is the tumor suppressor gene, which prevents the disorderly growth of cells and promotes the activation of the cell cycle checkpoint. Both proto-oncogenes and tumor suppressor genes can mutate and change their biological function. Such mutations cause the proto-oncogenes to become oncogenes and when they are overexpressed, they promote the unrestrained multiplication of cancer cells. On the other hand, mutated suppressor genes become inactive and low expressed, losing their ability to "brake" cell growth, decreasing their efficiency in fighting tumor progression (KIM *et al.*, 2020; LEE; MULLER, 2010). The same occurs in dogs with breast cancer, cancer cells in CMT also exhibit gene products

involved in tumor growth, such as overexpression of proto-oncogenes and a low expression of tumor suppressor genes. However, little is known about the mechanisms and molecules that contribute to the initial drives of cell division in CMT (Klopfleisch *et al.*, 2011).

Similar genetic alteration shared between CMT and HBC include the upregulation of oncogenes such as KRAS, PI3K/AKT and MAPK, as well as downregulation of tumor suppressors such as p16/INK4A, PTEN, BRCA1, and p53 (Fish *et al.*, 2018). Additionally, CMT has antagonistic expression levels of some HBC-related genes as for instance the SATB1 oncogene, which its increased expression is associated with poor prognosis HBC, while the opposite occurs in CMT, which shows the under expression of SATB1 expression was the opposite in CMT showing under expression (Klopfleisch *et al.*, 2011). Although there are differences in CMT and HBC molecular expressions, the disease is similar in many other aspects already discussed in this review.

Regarding cancer initiation, a heterogeneous subpopulation of cancer cells with stem-like properties has become evident in recent years. They are called cancer stem cells (CSCs) and have the ability to renew and differentiate, playing an important role not only in the initiation but also in the progression and metastasis of tumors. CSCs can originate from adult, progenitor, or somatic stem cells. Properties such as pluripotency cause CSCs to give rise to cancer cells with different phenotypes, resulting not only in the growth of the primary tumor but also in the appearance of new tumors (Chen; Huang; Chen, 2013; Hanahan; Coussens, 2012; Rybicka *et al.*, 2015). Furthermore, CSCs become immortal, as do most cancer cells due to the reversal of the natural telomere shortening process, and these cells have an incredible ability to resist chemotherapy, leading to disease remission (Chen; Huang; Chen, 2013; Pang; Argyle, 2010; Rybicka; Król, 2016). In HBC, CSCs with CD44+/CD24- phenotypes are associated with a poor prognosis, and the same is true in CMT. In dogs with mammary carcinomas, these CSCs phenotypes are associated with a poor prognosis, higher grade of carcinoma, and lymph node metastasis (Im *et al.*, 2015).

It is believed that after intense tumor progression, cancer cells lose cell cycle control and intercellular adhesion, entering the stromal and finally invading the peripheral blood and lymphatic vessels (da Costa *et al.*, 2012). These cells are called circulating tumor cells (CTC) and can originate from either the primary tumor or its metastases. In HBC, CTC are a prognostic factor for survival and predictive of short OS. These cell types are detectable in the blood by liquid biopsy in humans. This is a non-invasive method that can be used not only for cancer diagnosis but also for disease monitoring and to guide therapy (Marconato *et al.*, 2019; Von Bubnoff, 2017). It is known that the bone marrow is invaded by cancer cells

through blood flow and the presence of micrometastases at this site indicates that cancer cells circulate even in the pre-invasive stage of the disease, both in HBC patients and in animal models (Marconato *et al.*, 2019). One study reported that CRYAB is a potential marker of CTC in metastatic CMT and correlates with the vascular invasion of primary CMT (da Costa *et al.*, 2013). It is plausible, therefore, that CRYAB becomes an ideal biomarker for the detection of CTC in dogs with metastatic mammary tumors, but studies for its validation are still needed. The research on these biomarkers for CTC is very promising as there is a commercial appeal for veterinary practice due to the rapid and non-invasive collection of samples from animals with cancer.

2.1.6 Similarities between canine and human mammary tumor stroma

The mammary gland is a dynamic tissue derived from the epidermis, which reaches full maturity only after puberty. The development of the mammary gland and ducts depends on the interaction between epithelial and stromal cells (Humphrey; Dufresne; Schwartz, 2014). Mammary ducts are formed by luminal cells associated with myoepithelial cells surrounded by the basement membrane that separates the epithelium from the stromal (Kass *et al.*, 2007). The stromal modulates the development of the normal mammary gland, and in the presence of mammary neoplasms, the stromal also actively influences the malignancy of the tissue, favoring carcinogenesis (Klopfleisch, R. *et al.*, 2010). In addition to cancer factors from within the mammary gland itself, extracellular factors contribute to the creation of a microenvironment that can aid in the initiation, progression, and metastasis of mammary neoplasms (Hanahan; Weinberg, 2011).

Canine and human mammary tumors are complex and contain components that resemble the normal mammary gland, although it is structurally and functionally abnormal (Klopfleisch *et al.*, 2011). In addition to cancer cells, the stromal is composed of different cell types and an extracellular matrix (ECM) that are responsible for the heterogeneity of the tumor performing similar functions in normal tissues, such as support, stiffness, and substrates for cell growth. However, when these components are associated with tumors they control intercellular and cell-ECM adhesion, which in turn determine cancer cell invasion and metastasis (Gkretsi; Stylianopoulos, 2018; Mikala Egeblad, 2011). When stromal associates with cancer, it affects each of the "hallmarks of cancer" by sustaining cell proliferative signaling, evading growth suppressors, resisting cell death, enabling reproductive immortality,

inducing angiogenesis, activating invasion and metastasis, downregulating cellular energetics, and evading immune destruction (Hanahan; Coussens, 2012).

Fibroblasts synthesize connective tissue fibers as well as the proteoglycans and glycoproteins of the ECM, maintaining the integrity of the ECM as structural support for cells and organs. Fibroblasts are the main cells involved in the healing process. During tumorigenesis, activated fibroblasts are also called cancer-associated fibroblasts (CAFs) and influence the composition and architecture of the ECM. For instance, CAFs secrete signaling proteins that stimulate cancer cell proliferation such as mutagenic epithelial growth factors - hepatocyte growth factor (HGF), EGF family members, IGF-1, stromal cell-derived factor-1 (SDF-1/CXCL12), and others (Hanahan; Coussens, 2012). In HBC, CAFs are used to mark alpha-smooth muscle actin (αSMA), which is associated with reactive tumor stromal in HBC and other tumors. In canines, αSMA-positive cells are detected in adenomas and carcinomas and are significantly related to poor prognosis (Yoshimura *et al.*, 2015).

The ECM is responsible for storing factors that promote cell growth and provide a substrate for mammary gland development (Lu; Weaver; Werb, 2012). Additionally, the ECM is a crucial component of the stromal for tissue homeostasis and interacts closely with cancer cells for the transmission of signals from the cell via integrins. The integrins are responsible for binding cells to the ECM, aiding in the binding of cells to proteins, cytokines, proteases, and growth factors (Barczyk; Carracedo; Gullberg, 2010) such as TGF-β which when it is upregulated is responsible for the development of desmoplasia in tumors. Desmoplasia is an intense process of fibrous formation in the ECM presenting within the tumor with high levels of collagen, fibronectin, tenascin, and proteoglycans which are discussed next in more detail. In addition, desmoplasia increases the secretion and production of inflammatory factors and tumor growth factors, as well as abnormal proliferation of stromal cells. These features make desmoplastic tumors more aggressive and associated with worse prognosis in various types of cancer (Gkretsi; Stylianopoulos, 2018).

Collagen is an important component of the ECM and some human breast tumors exhibit intense collagen deposition (Walker, 2001). Collagen fibers can change their architecture during the process of carcinogenesis, transforming their helical shape to linear, making the tumor stromal more rigid. This transformation may aid cell invasion through collagen fibers or by increasing integrin signaling associated with ECM (Conklin; Keely, 2012). Type IV collagen is the main component of the basement membrane and the main barrier to tumor cell invasion (Gordon; Hahn, 2010). Canine mammary carcinoma cells show high cytoplasmic expression of type IV collagenase, which is responsible for collagen degradation,

aiding in tumor dissemination (PAPPARELLA, 1997). Another characteristic of CMT is the formation of different types of the matrix (metaplasia), such as bone, cartilaginous and myxoid matrices, which are present in both adenomas and carcinomas. On the other hand, mixed canine mammary tumors are characterized by varied matrix production that may contain different combinations of cartilage, bone tissue, adipose tissue, and fibrous tissue (Cassali *et al.*, 2014). However, for the purpose of studies for comparative oncology, mixed tumors are uncommon lesions in HBC, but are frequent in both human salivary glands and CMT (Genelhu *et al.*, 2007).

Fibronectin participates in the control of epithelial cell proliferation during acinar differentiation in mammary gland. During breast tumor formation, changes in ECM are accompanied by increased fibronectin production (Williams *et al.*, 2008). In both stromal and epithelial/myoepithelial cells of CMT, there is an increase in fibronectin expression, but this expression was not previously observed in regions of metastasis (Peña *et al.*, 1994). However, a recent proteomic study in CMT identified a high intensity of Fibronectin type III containing 1 protein (FNDC1) in not only tumors, but also highly expressed in regions of metastasis. High expression of FNDC1 in human patients is associated with a poor prognosis (Cordeiro *et al.*, 2021).

Tenascin is an ECM adhesion glycoprotein, similar to fibronectins, which interact with integrin receptors (Chiquet-Ehrismann; Chiquet, 2003). Tenascin is involved in tissue formation and regeneration, and it can be super expressed and related to tumor malignancy in HBC (Orend; Chiquet-Ehrismann, 2006). The presence of tenascin in tissues is increased in areas of ECM remodeling and neoplastic lesions, although it is CMT not associated with the malignant transformation of these tissues (Faustino *et al.*, 2002). It has also been observed that the association between tenascin and stromal myofibroblasts in CMT is correlated with high-grade breast carcinomas, indicating that tenascin may be related to tumor malignancy dependent on the type of cell producing it (Yoshimura *et al.*, 2015).

In recent years, studies have revealed that proteoglycans are essential macromolecules that affect cell functions through direct interaction with cell receptors and growth factors (Afratis *et al.*, 2012). Studies with mixed CMT have evaluated the presence of versican, a proteoglycan secreted into the ECM, whose expression has been found to increase with tissue remodeling of tumors, malignancy, and the tumor cells invasion (DE CAMPOS *et al.*, 2016; ERDÉLYI *et al.*, 2005; DAMASCENO *et al.*, 2012, 2014). In addition, in HBC and CMT it is known that proteoglycans in the stromal of malignant tumors and breast cancer

resistance protein are major actors in the multidrug resistance phenomenon, which remains a major challenge in the treatment of cancer (Levi *et al.*, 2021).

Apart from that, molecules that remodel the ECM, such as matrix metalloproteinases (MMPs) are also critical to the desmoplasia process. MMPs are responsible for degrading the matrix and are strongly controlled by tissue inhibitors of metalloproteinases (TIMPs). The inappropriate expression of these molecules leads to aberrant changes in the mammary gland (Fata; Werb; Bissell, 2004). A 10-fold higher expression of MMP-9 has been reported in CMT compared to the normal gland (Y *et al.*, 2001). In the same way, a high activity of MMP-2 and MMP-9 enzymes and low activity of TIMP-1, TIMP-2, and TIMP-3 has also been reported in CMT (Aresu *et al.*, 2011). Nevertheless, divergent results indicate that high TIMP-2 expression is related to malignancy, the increased metastatic potential of CMT, and a worse prognosis (Santos *et al.*, 2011).

2.1.7 Transcriptomics and proteomics analyses on CMT

In recent years, studies have investigated a limited number of molecular targets and biomarkers of CMT, mainly due to methodological limitations. Certainly, evaluation of tumor and stromal areas separately would be better for understanding how these areas interact in canine mammary tumors. Traditionally, immunohistochemistry and qPCR analyses are performed on bulk tumors and the results pertain to a conglomerate of different cell populations from both the tumor and the stromal area, without discriminating the molecular characteristics of each corresponding findings (Markkanen, 2019).

A research group demonstrated the existence of striking molecular homologies between CMT and HBC stromal tissues. Laser capture microdissection (LCM) was used to discriminate cancer-associated stromal (CAS) and normal stromal in FFPE subsections of canine simple mCA. Additionally, RT-qPCR was performed for analyzing the expression of 7 known human CAS markers to support canine CAS as a model for HBC. As result, proteins such as COLa1, αSMA, FAP, PDGFRβ, and CAV1 were significantly upregulated in canine CAS. This upregulation in canine CAS is consistent with findings in human studies. On other hand, it was found downregulation of CXCL12 in the canine CAS instead of being upregulated, as observed in many human studies (Ettlin *et al.*, 2017).

Subsequently, the same group characterized stromal reprogramming in canine simple mCA on a transcriptome scale using LCM-RNAseq. The number of downregulated genes that differed between normal and CAS was remarkable, and the majority of those genes

were correlated with ECM components, angiogenesis, immune system, cell adhesion, and differentiation. The gene expression in CAS supporting tumor growth and malignancy when compare stromal between human and canine mCA (Next-generation RNA sequencing of FFPE subsections reveals highly conserved stromal reprogramming between canine and human mammary carcinomaAmini *et al.*, 2019).

Following this in-depth characterization of stromal reprogramming in canine simple mCA, RNAseq analyses showed that the stromal in canine benign tumors are distinct from malignant ones and that changes exist between normal stromal, CAS in adenoma, and CAS in carcinomas. Such stromal genes between benign neoplasm and carcinomas as COL11A1, CD74, STRA6, HLA-DRA, VIT, PIGR, IGFBP4, and TNIP1 are involved in various hallmark-signaling pathways and at the level of cellular composition, and demonstrate their prognostic value for HBC. Also, these potential stromal modulators are important to understanding how stromal reprogramming works between benign and malignant CMT, in this way contributing more data to diagnostic and therapeutic purposes (Differential stromal reprogramming in benign and malignant naturally occurring canine mammary tumours identifies disease--promoting stromal componentsAmini *et al.*, 2019).

Indeed, CAS information in HBC and CMT at the transcriptomic level is extremely valuable and gives rise to several interesting questions to understand the extent to which transcriptional differences translate to the protein level. The studies previously mentioned in this session demonstrated significant changes at the transcriptional level between the CAS and normal stromal of canine simple mCA. Subsequently, proteomic analysis was performed in order to define such changes at the protein level and compare them with transcriptional changes in the same tissue. The most deregulated proteins were produced by stromal cells and the main protein expression alterations were associated with the cytoskeleton, extracellular matrix components (mainly collagens), and cytokines (mainly tumor necrosis factor). To strengthen canine CAS as a model for HBC, the researchers noticed that overexpression of POSTN, IGFBP2, IGFBP7, LTBP2, FN1, PLOD2, COL4A1, COL4A2, COL6A5, and COL12A1 in canine CAS they were related to poor OS in human breast cancer patients (Pöschel *et al.*, 2021).

Proteomics and mass spectrometry-based methods represent valuable tools for comparative oncology research. Recently, a study demonstrated that CANX, FNDC1, HSPA5, PDIA3, and A1BG were related to an immune response against tumor cells and metastatic potential in canine cancer. Higher expression of FNDC1 and A1BG was associated with shorter metastasis-free survival in human patients. These findings have been highlighted as a relevant

biomarker for various types of cancer in humans and represent a prognostic value for breast cancer in both dogs and humans. Additionally, peptides assigned to collagen types I, VI and XII had significant expression representing part of the crosstalk between stromal and cancer cells in female dogs diagnosed with metastatic breast cancer (Cordeiro *et al.*, 2021).

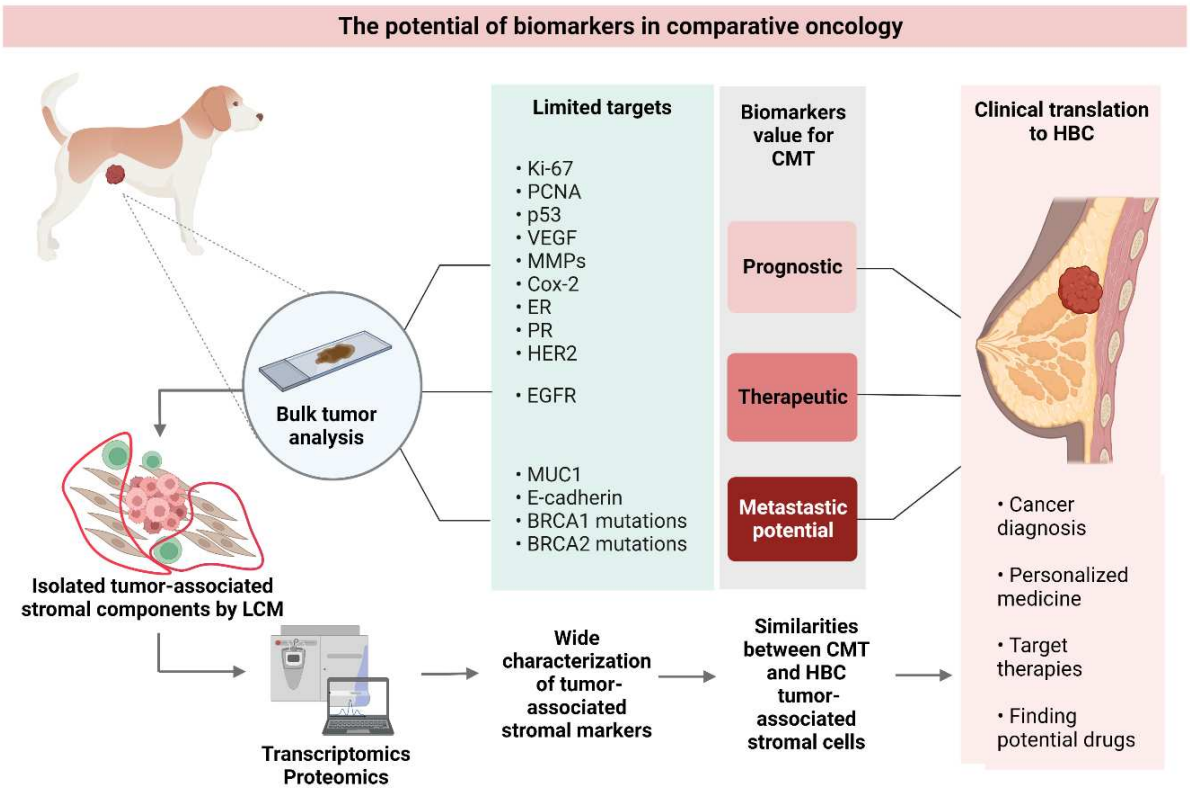
Studies about gene (Metastatic canine mammary carcinomas can be identified by a gene expression profile that partly overlaps with human breast cancer profilesKlopfleisch, Robert *et al.*, 2010) and protein (Proteome of metastatic canine mammary carcinomas: Similarities to and differences from human breast cancerKlopfleisch, Robert *et al.*, 2010) signatures suggest the existence of common underlying mechanisms occurring in metastatic CMT and HBC. A total of 1011 gene expression were identified in canine mammary carcinomas with lymph node metastases compared to canine non-metastatic carcinomas. Among metastatic carcinomas, overexpressed genes were associated with induction and maintenance of metastatic progression such as cell cycle regulation and matrix modulation. More than one-third of genes overexpressed in metastatic CMT compared with non-metastatic carcinomas were relevant for HBC (Metastatic canine mammary carcinomas can be identified by a gene expression profile that partly overlaps with human breast cancer profilesKlopfleisch, Robert *et al.*, 2010). The same research group also reported 21 differentially expressed protein were identified in canine mammary carcinomas with lymph node metastases compared to non-metastatic canine carcinomas. In addition, 11 up-regulated proteins in metastatic CMT were related to cell proliferation, cell motility, regulators of matrix invasion, and protection against hypoxia. Most of these proteins are also dysregulated in human cancer (Proteome of metastatic canine mammary carcinomas: Similarities to and differences from human breast cancerKlopfleisch, Robert *et al.*, 2010)

Proteomics applications in comparative oncology have greatly increased in recent years, mainly in studies related to the diagnosis and pathogenesis of tumor cells. Gel-free high throughput proteomics is used for a better comprehension in molecular pathogenesis and prospective biomarker research. However, microarrays and two-dimensional differential gel electrophoresis (2DGE) are still a major part of applications in canine oncology and they should be complementary to gel-free proteomic (Ceciliani *et al.*, 2016). By applying 2DGE and MALDI-TOF-MS (laser-assisted desorption time-of-flight/mass monitoring), (Klose *et al.*, 2011) identified 48 proteins with differentially expression during malignant progression from normal mammary gland to benign tumor formation (adenoma), following to malignant tumor formations (non-metastatic carcinoma and metastatic carcinoma) in dogs. Interestingly, no details are yet known about tumor malignant progression and whether it is associated with a

linear change in protein expression in breast tumors in any species. In conclusion, the study showed that protein expression is associated with the tumor malignancy process in a gradual way rather than in a progressive way, and the major protein expression changes were identified in canine mammary metastatic carcinomas.

Canines have made great contributions to our understanding of cancer biology and to the search for efficient treatments for breast cancer. Studying spontaneous breast cancer in dogs as well as characterizing tumor cells and stromal through state-of-the-art techniques such as transcriptomics and proteomics give us valuable information that optimizes comparative oncology. In recent years, the demand for breast cancer biomarkers for diagnosis and prognosis in pets has been very active, but an ideal and easily applicable biomarker has not yet been found in the veterinary routine. A deeper investigation of tumor stromal in the diagnosis of CMT becomes important to establish the relationship between stromal components and neoplastic cells, besides providing data about the biological behavior and clinical staging of CMT and thereby favoring therapeutic approaches targeted to the patient (Figure 2). Studies in CMT have been provided molecular targets with biomarker potential in comparative oncology. Analysis of bulk tumors contributes to biomarker investigation, but offers a limited number of targets and reflect alterations either to the tumor cells or to the stromal cells. To complement and enhance our understanding of the components of isolated stromal reprogramming in CMT, transcriptomics and proteomics analyses provide a wide characterization of stromal reprogramming markers that allows us to understand how CMT and HBC are comparable in all their aspects.

Figure 2 - The potential of biomarkers in comparative oncology.



2.2 Conclusion

In recent years, the search for biomarkers for the diagnosis and prognosis of canine mammary tumors has been very active however, an ideal and easily applicable biomarker has not been identified in the veterinary routine yet. A further investigation of the tumor stroma in the diagnosis of CMT becomes relevant to establish the relationship between stromal components and neoplastic cells, in addition to providing data on the biological behavior and clinical staging of CMT, thus favoring patient-directed therapeutic approaches. The study of spontaneous breast cancer in dogs as well as the characterization of tumor cells and stroma using state-of-the-art techniques such as transcriptomics and proteomics, reinforce that CMT can be used as a model for HBC, evaluating the mechanisms involved in breast cancer development and progression.

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3 CHAPTER 2: PATHOBIOLOGY AND PROTEOME OF CANINE MAMMARY TUMOR: A COMPARATIVE ONCOLOGY APPROACH

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PATOBIOLOGIA E PROTEOMA DE TUMORES MAMÁRIOS CANINOS: UMA ABORDAGEM PARA A ONCOLOGIA COMPARATIVA

RESUMO

Tumores mamários espontâneos em cães têm sido destacados como um modelo importante para o câncer de mama humano contribuindo para a compreensão da tumorigênese, progressão e mecanismos de metástase. Em busca de candidatos a biomarcadores de estado de doença, usamos a abordagem proteômica para detectar alterações moleculares, comparando a expressão de proteínas de tumores mamários caninos (CMT) e glândulas mamárias normais (NT). Foram coletadas amostras de tecidos (5 NT e 5 CMT) obtidas de cadelas post-mortem do Centro de Controle de Zoonoses (CCZ). As amostras foram divididas em duas porções. Uma porção foi congelada e armazenada em -17 ◦C até ser utilizada para análise proteômica e a outra porção seguiu para os procedimentos padrão de biópsia e foi armazenada à temperatura ambiente. Tumores mistos benignos (n=2), carcinoma inflamatório (n=1), adenomas complexos (n=2), NT (n=5) foram selecionados para a abordagem proteômica em shotgun. A pesquisa de espectro peptídico foi realizada usando Progenesis v4.2 no qual permitiu a identificação em média de 945 proteínas, sendo 136 proteínas super expressas em CMT. A maioria dessas proteínas super expressas estava envolvida em processos biológicos como translocação de SLC2A4 (GLUT4) para a membrana plasmática, dobragem de proteínas, glicólise e gluconeogênese, e complexo de maturação da kinase 1. No estudo, FN1, FGD3, TNN, ACAN, e ATP5F1B como as proteínas mais abundantes dentre as proteínas super expressas dos CMT. Os genes foram selecionados para modulação potencial por miRNAs e identificou, principalmente, miRNAs ligados a proliferação celular e remodelação de matriz extracelular. Finalmente, uma validação independente mostrou que ATP5F1B pode ser um candidato a biomarcador de prognóstico para doentes humanos com câncer da mama. Assim, através de uma caracterização molecular de tumores mamários espontâneos, descrevemos proteínas-chave que podem ser ainda úteis na validação de biomarcadores.

Palavras-chave: proteômica, shotgun, oncologia comparativa, tumores mamários caninos.

PATHOBIOLOGY AND PROTEOME OF CANINE MAMMARY TUMOR: A COMPARATIVE ONCOLOGY APPROACH

ABSTRACT

Spontaneous mammary tumors in dogs have been highlighted as an important model for human breast cancer contributing to the understanding of tumorigenesis, progression and metastasis mechanisms. In search of candidate disease state biomarkers, we used the proteomics approach to detect molecular changes by comparing protein expression of canine mammary tumors (CMT) and normal mammary glands (NT). Tissue samples (5 NT and 5 CMT) obtained from post-mortem female dogs from the Zoonosis Control Center (CCZ) were collected. The samples were divided into two portions. One portion was frozen and stored at -17 ◦C until used for proteomic analysis and the other portion followed standard biopsy procedures and was stored at room temperature. Benign mixed tumors (n=2), inflammatory carcinoma (n=1), complex adenomas (n=2), NT (n=5) were selected for the shotgun proteomic approach. Peptide spectrum search was performed using Progenesis v4.2 in which allowed the identification of an average of 945 proteins, 136 of which were overexpressed proteins in CMT. Most of these overexpressed proteins were involved in biological processes such as translocation of SLC2A4 (GLUT4) to the plasma membrane, protein folding, glycolysis and gluconeogenesis, and maturation complex kinase 1. In the study, FN1, FGD3, TNN, ACAN, and ATP5F1B as the most abundant proteins among the overexpressed proteins of CMT. The genes were screened for potential modulation by miRNAs and mainly identified miRNAs linked to cell proliferation and extracellular matrix remodeling. Finally, an independent validation showed that ATP5F1B may be a candidate prognostic biomarker for human breast cancer patients. Thus, through a molecular characterization of spontaneous breast tumors, we describe key proteins that may be further useful in biomarker validation.

Keywords: proteomics, shotgun, comparative oncology, canine mammary tumors.

3.1 Introduction

According to GLOBOCAN, human breast cancer (HBC) contributed to 30.3% of the prevalent cancer cases in women over the past five years and accounted for 15.5% of deaths among women in 2020 (Sung *et al.*, 2021). Unfortunately, cancer incidence has also increased in canines in recent years, and mammary tumors are the most common type of tumor in intact female dogs, accounting for nearly 50% of all tumors ((Fonseca; Daleck, 2000)Fonseca, 2000). In addition to the high incidence of mammary tumors in both women and bitches, spontaneous canine mammary tumors are an excellent example of the complex pathobiology of cancer behind the heterogeneous course of this disease in humans. Furthermore, dogs not only share the same environment with humans being equally exposed to external pollutants, but they also share anatomical and histological similarities, metastatic tumor patterns, equivalent clinical course, as well as more homologous DNA and protein sequences compared to murine models (LIU *et al.*, 2014; MARCONATO *et al.*, 2019; MILES *et al.*, 2008; PAOLONI; KHANNA, 2008; ROWELL; MCCARTHY; ALVAREZ, 2011). In this context, improve in veterinary care and the longer life expectancy of dogs, it is possible to understand how canine tumors are similar to human tumors, thus reinforcing these animals as a reliable model for comparative oncology.

Proteins are essential components of cells, as they are required to drive cellular activities. In this regard, the study of proteins is essential to understand the biological mechanisms of tumor cells, as well as the protein-protein networks that lead to breast cell dysregulation and cancer progression (Hondermarck *et al.*, 2008; Liu *et al.*, 2014). Therefore, breast cancer proteomics has already provided significant data in terms of proteome signatures, in addition to the identification of some proteins with potential interest for diagnosis and treatment. Many of the techniques used in proteomics focus on the identification of biomarkers, which contribute not only to obtaining a diagnosis, but also assist in individualized therapies and monitoring therapeutic efficacy. Although progress has been slow, the prospect of individualized treatment based on the tumor proteome could become a reality in the near future (Hondermarck *et al.*, 2008; Miles *et al.*, 2008; Morris, 2016).

One of the most widely used MS-based proteomics approaches consists of a bottom-up analysis where proteins are first extracted from the tissue sample and then enzymatically digested into corresponding peptides, which are separated by liquid chromatography (LC) and analyzed by mass spectrometry (MS) (Gromov; Moreira; Gromova, 2014). This approach uses a highly accurate determination of the corresponding masses of the

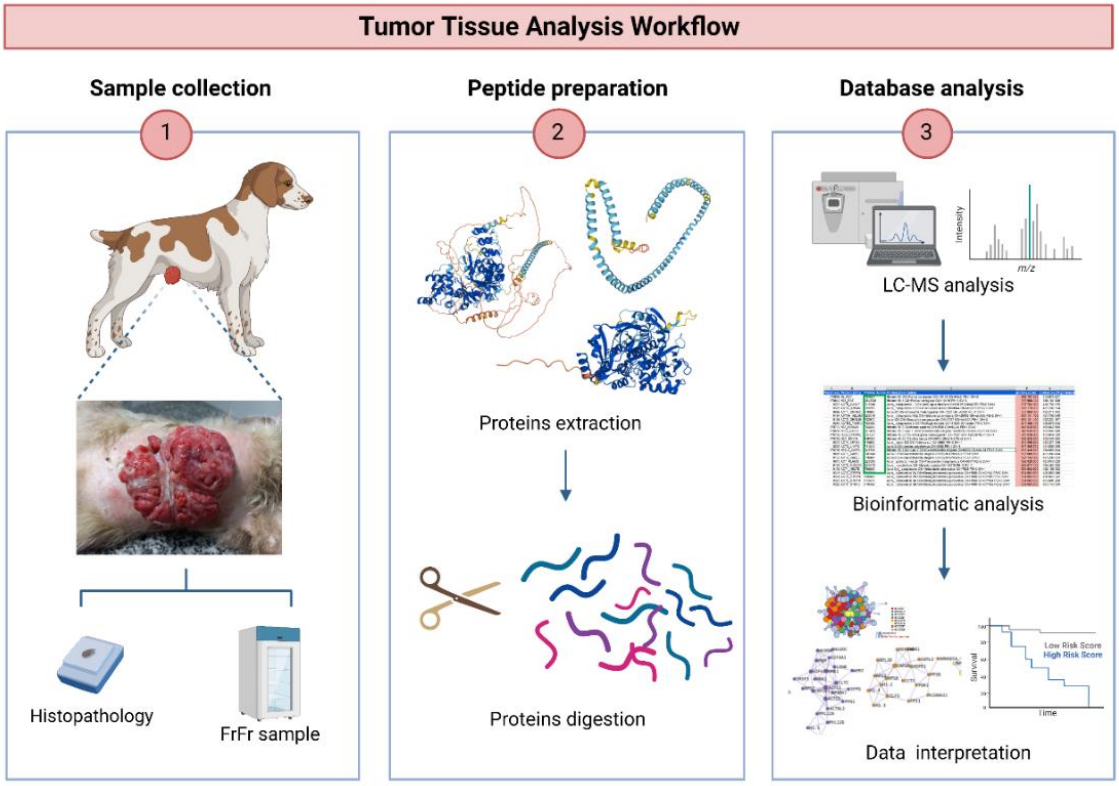
target peptide sequences obtained by MS, which are combined with known protein databases. In this way, it is possible to identify relevant proteins that represent a valuable tool for basic and translational research in oncology. Thus, the present study aims to perform a comparative and quantitative analysis of proteins from canine mammary tumors (CMT) using bottom-up proteomics to provide insights into molecular pathophysiology of the disease.

3.2 Materials and methods

3.2.1 Study design

In the present study, label-free data-dependent acquisition (*DDA*) mass spectrometry was used to decipher the proteome of CMT (n = 5) and NT (n = 5) from surgical excision in bitches euthanized at the CCZ in Fortaleza, Brazil. Then, the samples were transported to the laboratory within 1 h postmortem and were divided into two portions. One portion was frozen and the other portion was followed for standard biopsy procedures. Bioinformatics and statistical tools were used to identify and describe differentially expressed proteins between CMT and non-tumor tissues. We intend to study the connection between gene expression of such proteins and clinical findings in HBC patients using the Kaplan- Meier online database (Figure 3). The first step of the workflow was the sample collection, which began with the bulk tumor macrodissection followed by the division of the sample into two portions (histopathology and fresh-frozen). The second step was the peptide preparation, which comprises two main procedures (extraction and digestion). The last step consisted of LC-MS database analysis, followed by bioinformatics analysis and data interpretation.

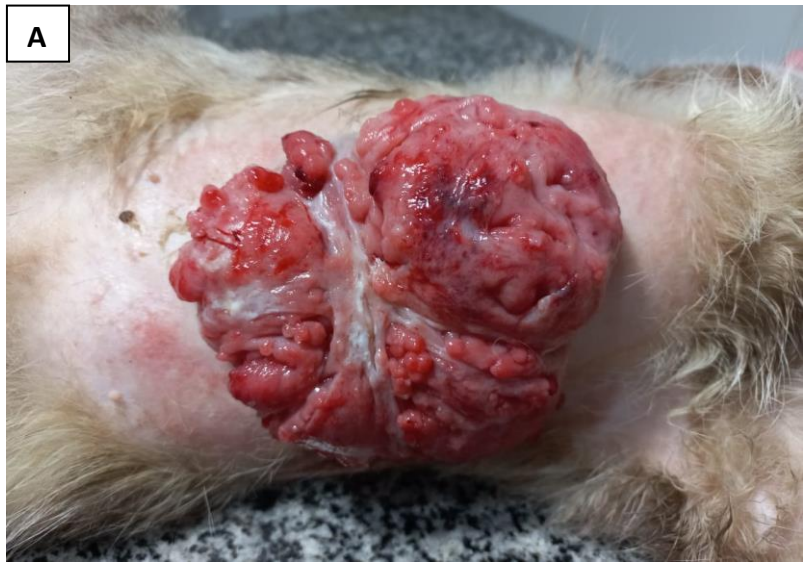
Figure 3 - Tumor tissue analysis workflow.



3.3.2 Tissue samples

We analyzed five canine mammary tumors (CMT) and five normal mammary glands tissues (NT) obtained from post-mortem female dogs in euthanasia routine from CCZ (Fortaleza, Brazil). All tissue samples were kept on ice immediately after surgery to prevent proteolytic degradation and were subsequently transported to the laboratory within 1 h after surgery. Tissue sections were cut from macroscopically visible tumor areas in order to histologically confirm the presence of cancer. Similarly, normal mammary gland tissues also underwent the same histological procedure. The tissue samples were then divided into two portions. One portion was frozen and stored at -20 ◦C (Fresh-frozen FrFr) until used for proteomics. The other portion was fixed in formalin, followed by standard biopsy procedures, and it was stored at room temperature. Benign mixed tumors (n=2), Inflammatory carcinoma (n=1), complex adenomas (n=2), and normal mammary gland tissues (n=5) were selected for the proteomics approach (Figure 4).

Figure 4 - A) Mammary tumor in a female dog (post-mortem). Time of sample collection from the mammary tumor and from the normal mammary gland. B) Formalin-fixed and paraffin-embedded (FFPE) samples of normal glands (above) and canine mammary tumors (below). C) Tissue sections were cut from macroscopically visible tumor areas to histologically confirm the diagnostics.

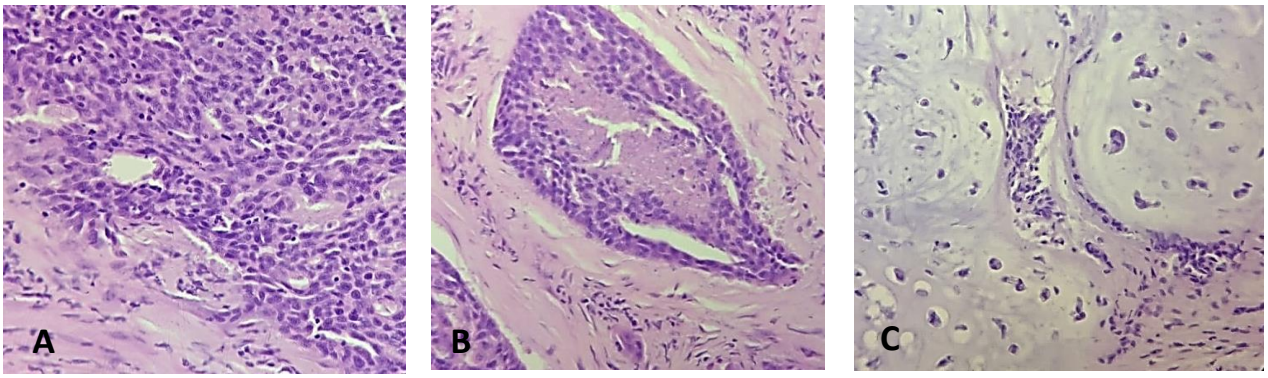


	Diagnostic
MT1	Benign Mixed
MT2	Complex Adenoma
MT3	Inflammatory Carcinoma
MT4	Complex Adenoma
MT5	Benign Mixed

3.2.3 Histopathologic evaluation

For histopathologic study, tumor specimens were fixed in a 10% neutral buffered formalin for 48–56 h at room temperature (RT), dehydrated in a graded series of ethanol, clarified with xylene, embedded in paraffin wax, and serially sectioned into 3 μm thickness. The sections were stained with hematoxylin and eosin. The tumors were classified according to the World Health Organization (WHO) classification system of canine mammary tumors (MISDORP, 1999). The diagnosis of mammary cancer was confirmed by histological evaluation as defined by (Goldschmidt *et al.*, 2011). Likewise, normal gland mammary tissues were also evaluated to confirm as healthy controls.

Figure 5 - Tubular carcinoma (A) and areas of central necrosis in neoplasia (B) of MT3 sample. Cartilage matrix in canine benign mixed tumor (C).



3.2.4 Sample preparation, protein extraction, trypsinization and desalting

A total of 50 mg of tissue per sample was subjected to protein extraction with 300 μl of Lysis buffer containing 8 M urea, 0.075 M NaCl, 0.05 M Tris, 0.2 M sodium orthovanadate, 0.2 M beta-glycerophosphate and protease inhibitor cocktail at a concentration of 1:100, followed by shaking on ice for 15 minutes. The samples were then sonicated three times for 1 minute, with 2-minute intervals between each sonication. After this stage, the sample was centrifuged for 15 minutes at 4ºC and 14.000 rpm. The supernatant was then transferred to clean tubes and stored at -20ºC. The protein concentration in all samples was measured using the Bradford method (BRADFORD, 1976) in duplicates against a standard curve of bovine serum albumin (BSA) and stored at -20 ◦C until use.

Extracted proteins (60 µg) were used in the reduction process, which a volume of dithiothreitol added to reach a final concentration of 0.005 M. This mixture was kept at 56°C in a water bath for 25 minutes. Subsequently, as an alkylation process, a volume of iodoacetamide was added to reach a final concentration of 0.014 M, being kept in a dark environment for 15 minutes. The sample was diluted in ammonium bicarbonate to reduce the concentration of urea from 8 M to 1.6 M, and immediately afterwards, a volume of buffer was added to reach a final concentration of 0.001 M CaCl2. All samples were digested with trypsin with an enzyme/substrate ratio of 1/50 (w / w) and incubated at 37°C in a water bath for 16 h. A solution of trifluoroacetic acid (TFA) was added to a final concentration of 0.4% to interrupt tryptic activity.

SepPak TC18 50mg 1cc columns (Waters, USA) were coupled to a Visiprep SPE Vacuum Manifold (Sigma Aldrich, USA) for peptide desalting (Otávio *et al.*, 2023). Briefly, to activate the columns, 3 ml of acetonitrile were added in order to wash and activate the C18 column present in the SepPak. Then, the columns were equilibrated with 1 ml of 0.1% formic acid and a second step to balance the columns was performed with 3 ml of 0.1% trifluoroacetic acid. The previously digested samples were loaded into the column and washed with 3 ml of 0.1% trifluoroacetic acid to remove the salt. After washing, the columns were equilibrated with 1 ml of 0.1% formic acid, followed by elution with a solution containing 50% acetonitrile and 50% 0.1% formic acid. A final elution was performed with a solution containing 80% acetonitrile and 20% 0.1% formic acid. The final volume was lyophilized and stored at -80ºC until further analysis. Before mass spectrometry analysis, the samples were subjected to peptide quantification (QubitTM; Thermo Fisher, Waltham, MA, USA).

3.2.5 Label-free mass spectrometry

The experiments were performed in a Synapt XS mass spectrometer (Waters ®), coupled to a UPLC M-Class chromatography system. Chromatography was performed on a 130 Å pore size reversed phase column with a gradient from 0% to 40% (v/v) acetonitrile, containing 0.1% formic acid at 500 nL/min on a M-Class UPLC system. The ACQUITY UPLC column is 300 µm × 150 mm and 200 ng of sample, containing the peptides obtained from the tryptic digestion, were applied. For all measurements the mass spectrometer was operated in 'V' mode, with a resolution power of at least 12,000 m/z. All analyses were performed using electrospray ionization in ESI (+) mode using the microLockSpray source. The collection channel of the analyzed sample was closed every 30 sec for passage of the reference ion. The

mass spectrometer was calibrated with a solution of GFP ([Glu]-fibrinogen peptide) 500 fmol/mL injected through the reference spray from the microLockSpray source. The doubly charged ion ([M +2H]2+) was used for single-point initial (Lteff) calibration, and MS/MS ion fragmentation of GFP was used to obtain the final calibration of the instrument.

3.2.6 Database search, data analysis, and protein identification

All samples were applied in triplicate, and protein identification and analysis were generated using dedicated species-specific database search algorithms. MassLynx v4.1 software was used for data collection for processing and Progenesis v4.2 software was used for searching the appropriate database. The databases UniProtKB/Swiss-Prot 57.1 and UniProtKB/TrEMBL 40.1 were used and the search conditions were based on the taxonomy of the species worked on here. The comparisons were made between the mean ± standard error of the mean values of the readings of each subset of samples, such as the samples of tumors and non-tumors. The Progenesis QI V2.0/TransOmics Informatics (Waters Corporation) software was used to process and search the data using the principle of the search algorithm. The data were filtered to show only statistically significant [$P<0.05$; analysis of variance (ANOVA) and post hoc Tukey's test] changes in protein concentration with maximum fold change (MFC) ≥1.5. Normalized label-free quantification was achieved to plot principal component analysis against data split into two groups. Hierarchical Cluster Analysis of the expression profiles of the test samples between all (tumors and non-tumors) was conducted and Bray Curtis Correlation distance metric and an average linkage clustering method from the J-Express Pro V1.1 software (java.sun.com) was used to generate dendrograms. $P\leq0.05$ was considered to indicate a statistically significant difference. For classification of the identified proteins, the UniProt database (https://www.uniprot.org/) was used.

3.2.7 Gene ontology, in silico analysis of protein functional clusters

For a wider understanding of protein function, the human genome coding database was used as a reference set. Functional enrichment analysis was performed using associations of Metascape branded gene set collections (https://metascape.org/) (Zhou *et al.*, 2019). Default settings were applied (hypergeometry $p < 0.01$, ≥3 molecules assigned, and an enrichment factor >1.5).

Networks of FN1, FGD3, TNN, ACAN, and ATP5F1B proteins were analyzed using String platform version 11.5 (https://string-db.org/) (Szklarczyk *et al.*, 2019). Protein physical interactions, network type, network edges, and active interaction sources were based on String default (settings selected for *Canis lupus familiaris*) was used; a required confidence (combined score) > 0.9 was set as the cutoff criterion.

Genes defined with gene ontology terms were screnned for a modulation by miRNAs. Potential genes were analyzed for miRNA targets using *Homo sapiens* dataset of miRBAse (http://www.mirbase.org) (KOZOMARA; GRIFFITHS-JONES, 2011), once the search mechanisms were not available for *Canis lupus familiaris* miRNA data. To obtain the interaction between miRNAs and genes, data were submitted to miRNet 2.0 server (https://www.mirnet.ca/) (Chang *et al.*, 2020).

3.2.8 Biomarker comparative validation

To more closely evaluate the relevance of significant proteins found in canine mammary tumors as prognostic predictors in human breast cancer, an *in silico* validation was performed using The Human Protein Atlas (https://www.proteinatlas.org/). The main goal of this tool is a meta-analysis-based discovery and validation of survival biomarkers for cancer research. Overall survival (OS) was analyzed using protein IDs as input, and the patients were divided by best cutoff. One restriction was made regarding gender (female patients only), but no one restriction was made regarding tumor stage. It is noteworthy that the observed The Cancer Genome Atlas (TCGA) data are based on mRNAs expression levels, which under consideration of a putative post-transcriptional or post-translational gene regulation might not reflect the in vivo protein-based result. Survival curve, number of patients, and log-rank p (p score) were shown in the Kaplan-Meier plot. Log-rank p showing results for analysis of correlation between mRNA expression level and patient survival and it was considered a significant marker when p<0,001.

3.3 Results

3.3.1 Proteomic Analysis of CMT and NT tissues

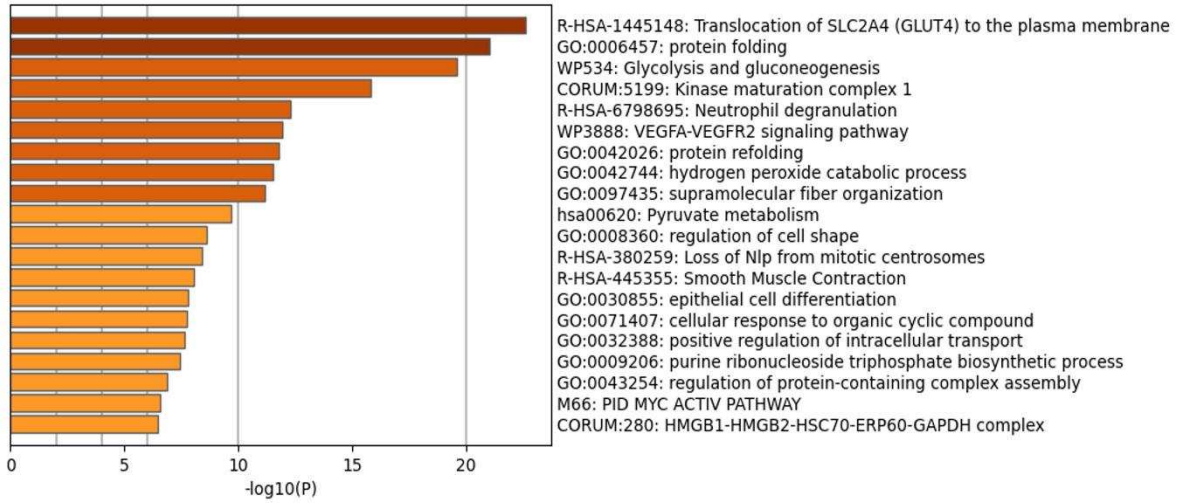
As determined by LC-MS/MS, a list containing 945 proteins was obtained from all tissues examined. To increase confidence in the detected differences, only those proteins with different abundances ($p < 0.05$) between CMT and NT tissues were considered. Following these criteria and by removing contaminating proteins, and a minimum of 1 unique peptide per protein was required for identification, a total of 204 proteins were considered for subsequent evaluation. The latter included 136 and 68 proteins that were up- and down-regulated in the CMT compared to the NT, respectively.

3.3.2 Pathway and Process Enrichment Analysis

Enrichment analysis of biological processes for 136 up-regulated proteins of CMT were performed a pathway analysis and process enrichment were performed according to Metascape analysis (the latest version of the database updated on 2022/11/28). A heat map illustrates the top 20 clusters based on gene ontology analysis that shows the main biological processes related to the up-regulated proteins in CMT (Figure 5A). The intensity of the bar color indicates the statistically significant p-values. Additionally, the analysis indicates that majority of proteins was involved in translocation of SLC2A4 (GLUT4) to the plasma membrane, protein folding, glycolysis and gluconeogenesis, and kinase maturation complex 1. Each cluster and the percentage of the genes found associated with each gene ontology term (Table 2). To annotate biological processes in which these proteins are involved, enrichment gene ontology (GO) analyses of these proteins were exploited (Table 3).

Figure 6 - A) Heat map derived from GO enrichment analysis of up-regulated proteins in CMT with a five-fold change or greater. B) Heat map derived from GO enrichment analysis of down-regulated proteins in CMT with a five-fold change or greater.

A



B

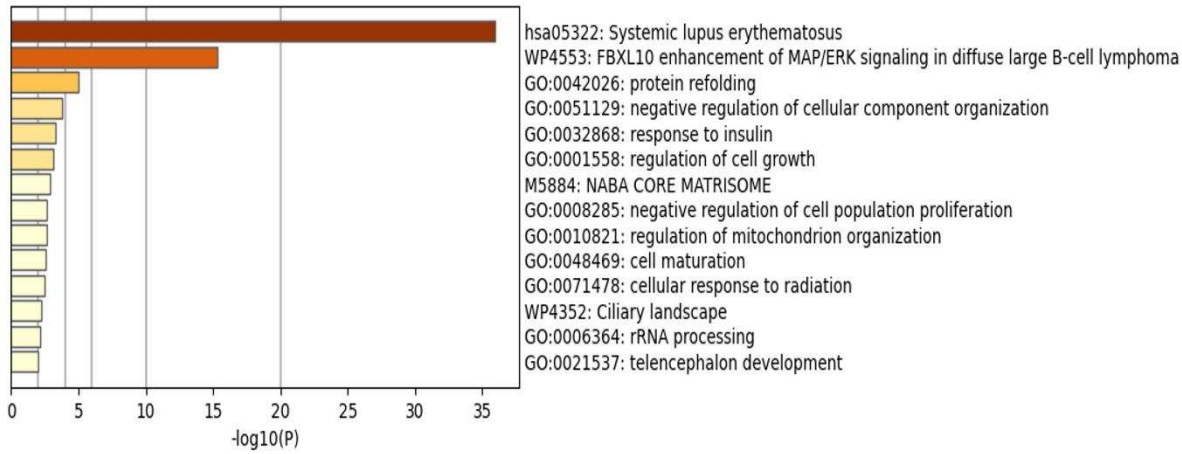


Table 2 - Top 20 clusters with their representative biological signal pathway and biological process enrichment evaluation associated with the up-regulated proteins in CMT.

	GO	Category	Description	Count	%	Log10(P)	Log10(q)
1	R-HSA-1445148	Reactome Gene Sets	Translocation of SLC2A4 (GLUT4) to the plasma membrane	15	14.85	-22.61	-18.42
2	GO:0006457	GO Biological Processes	protein folding	19	18.81	-21.00	-17.13
3	WP534	WikiPathways	Glycolysis and gluconeogenesis	12	11.88	-19.58	-16.13
4	CORUM:5199	CORUM	Kinase maturation complex 1	8	7.92	-15.82	-12.82
5	R-HSA-6798695	Reactome Gene Sets	Neutrophil degranulation	17	16.83	-12.29	-9.75
6	WP3888	WikiPathways	VEGFA-VEGFR2 signaling pathway	16	15.84	-11.90	-9.41
7	GO:0042026	GO Biological Processes	protein refolding	7	6.93	-11.76	-9.29
8	GO:0042744	GO Biological Processes	hydrogen peroxide catabolic process	7	6.93	-11.49	-9.06
9	GO:0097435	GO Biological Processes	supramolecular fiber organization	17	16.83	-11.18	-8.77
10	hsa00620	KEGG Pathway	Pyruvate metabolism	7	6.93	-9.67	-7.39
11	GO:0008360	GO Biological Processes	regulation of cell shape	9	8.91	-8.58	-6.41
12	R-HSA-380259	Reactome Gene Sets	Loss of Nlp from mitotic centrosomes	7	6.93	-8.41	-6.26
13	R-HSA-445355	Reactome Gene Sets	Smooth Muscle Contraction	6	5.94	-8.05	-5.91
14	GO:0030855	GO Biological Processes	epithelial cell differentiation	14	13.86	-7.80	-5.69
15	GO:0071407	GO Biological Processes	cellular response to organic cyclic compound	13	12.87	-7.76	-5.65
16	GO:0032388	GO Biological Processes	positive regulation of intracellular transport	9	8.91	-7.61	-5.51
17	GO:0009206	GO Biological Processes	purine ribonucleoside triphosphate biosynthetic process	7	6.93	-7.45	-5.36
18	GO:0043254	GO Biological Processes	regulation of protein-containing complex assembly	11	10.89	-6.87	-4.83
19	M66	Canonical Pathways	PID MYC ACTIV PATHWAY	6	5.94	-6.56	-4.54
20	CORUM:280	CORUM	HMGB1-HMGB2-HSC70-ERP60-GAPDH complex	3	2.97	-6.44	-4.43

"Count" is the number of genes in the user-provided list with membership in the given ontology term. "%" is the percentage of all of the user-provided genes that are found in the given ontology term. "Log10(P)" is the p-value in log base 10. "Log10(q)" is the multi-test adjusted p-value in log base 10.

Table 3 - Enrichment gene ontology analysis of biological processes for 136 up-regulated proteins of CMT identified by LC/MS-MS.

Biological Process*	GO- Identified proteins involved in process	Logp
Translocation of SLC2A4 (GLUT4) to the plasma membrane	SFN, TUBB2A, YWHAB, YWHAE, YWHAG, YWHAH, YWHA, TUBB3	-2,26054E+16
Protein folding	TUBB2A, TUBB3, TUBB4A, TUBB4B, TUBB1, TUBB6, TUBB2B	-7,41656E+15
Glycolysis and gluconeogenesis	ALDOA, GAPDH, LDHA, LDHB, LDHC, MDH2, PGK1, PGK2, PKLR, PKM, TPI1	-1,95775E+16
Kinase maturation complex 1	HSP90AA1, HSP90AB1, YWHAB, YWHAE, YWHAG, YWHAH, YWHAZ, YWHAQ	-1,5825E+16
Neutrophil degranulation	ALDOA, CTSD, HBB, HP, HSPA8, HSP90AA1, HSP90AB1, LTF, LYZ, MPO, NME2, PKM, PRDX6, TUBB4B, TUBB	-1,22948E+16
VEGFA-VEGFR2 signaling pathway	ALDOA, ANXA1, GAPDH, HBD, HSP90AA1, LDHA, PFN1, PGK1, RPLP2, PRDX2, TPM3, EZR, YWHAE, PRDX6, TUBB8	-1,19039E+16
Protein refolding	FKBP1A, HSPA5, HSPA8, HSPE1	-1,17581E+16
Hydrogen peroxide catabolic process	HBB, HBD, HBE1, HBG1, HBG2, MPO, PRDX2	-1,1494E+16
Supramolecular fiber organization	ACTN1, ALDOA, CAPG, FKBP1A, LNA, HSP90AB1, KRT8, KRT81, KRT83, KRT85, KRT86, TPM2, TPM3, HSP90B1, EZR, AKAP9, MYL9	-1,11753E+15

The Metascape was applied to functionally annotate enriched proteins, using the ontology sources. All genes in the genome have been used as the enrichment background. Terms with a p-value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 (the enrichment factor is the ratio between the observed counts and the counts expected by chance) are collected and grouped into clusters based on their membership similarities. More specifically, p-values are calculated based on the cumulative hypergeometric distribution, and q-values are calculated using the Benjamini-Hochberg procedure to account for multiple testings. Kappa scores are used as the similarity metric when performing hierarchical clustering on the enriched terms, and sub-trees with a similarity of > 0.3 are considered a cluster. The most statistically significant term within a cluster is chosen to represent the cluster.

Besides a general overview of GO processes, we also performed a functional pathway enrichment of differentially expressed proteins among the main biological processes. Some of the up-regulated proteins in CMT were highlighted among the eight biological processes shown in Table 3. Beta tubulins belonging to the same family/type (TUBB2A,

TUBB3, TUBB4A, TUBB4B, TUBB1, TUBB6, TUBB2B, TUBB, and TUBB8), whose absolute intensity was higher in CMT, were significant for four distinct pathways, such as translocation of SLC2A4 (GLUT4) to the plasma membrane, protein folding, neutrophil degranulation, and the VEGFA-VEGFR2 signaling pathway. In addition, the fructose-bisphosphate aldolase (ALDOA) showed the third highest biological process among all significant pathways as glycolysis and gluconeogenesis, and also played an important role in neutrophil degranulation. In particular, ALDOA also was significant for the VEGFA-VEGFR2 signaling pathway and supramolecular fiber organization. The heat shock protein 90-alpha family (HSP90AB1, HSP90AA1, HSP90B1) was significant for four different pathways such as maturation kinase complex 1, neutrophil degranulation, VEGFA-VEGFR2 signaling pathway, and supramolecular fiber organization. However, the latter protein family was found to be less intense in CMT compared to the tubulin and fructose-bisphosphate aldolase families. A notable participation of the 14-3-3 family (YWHAB, YWHAE, YWHAG, YWHAH, YWHA) and the heat shock protein 70 family (HSPA5, HSPA8, HSPE1) were also signed between the biological pathways.

Regarding the biological process enrichment analysis for 68 proteins downregulated in CMT compared to NT, the main biological pathways were involved in systemic lupus erythematosus, FBXL10 enhancement of MAP/ERK signaling in diffuse large B-cell lymphoma, protein refolding, negative regulation of cellular component organization, insulin response, cell growth regulation, naba core matrisome, and negative regulation of cell population proliferation (Figure 5B, Table 4). Some of the down-regulated proteins in CMT were outstanding among the biological processes such as the proteins belonging to the histone H2A family (H2AC8, H2AC7, H2AX, H2AC13, H2AC14, H2AC16, H2AC6, H2AC18, H2AC20, H2AJ, H2AC12, H2AC25, H2AX) and histone H3 family (H3-3A, H3-4, H3C1, H3C6, H3C12, H3C14, H3C15, H3-5, H3C14). These families are involved in five biological processes such as systemic lupus erythematosus, FBXL10 enhancement of MAP/ERK signaling in diffuse large B-cell lymphoma, negative regulation of cellular component organization, regulation of cell growth, and negative regulation of cell population proliferation. The heat shock 70 protein family (HSPA1A and HSPA1L) was also prominent among four biological processes, such as protein refolding, negative regulation of cellular component organization, regulation of cell growth, and negative regulation of cell population proliferation. To annotate biological processes in which these protein families are involved, enrichment gene ontology analyses of these proteins were exploited (Table 5).

Table 4 - Top clusters with their representative biological signal pathway and biological process enrichment evaluation associated with the down-regulated proteins in CMT.

	GO	Category	Description	Count	%	Log10(P)	Log10(q)
1	hsa05322	KEGG Pathway	Systemic lupus erythematosus	21	41.18	-35.96	-31.61
2	WP4553	WikiPathways	FBXL10 enhancement of MAP/ERK signaling in diffuse large B-cell lymphoma	8	15.69	-15.30	-12.80
3	GO:0042026	GO Biological Processes	protein refolding	3	5.88	-4.99	-2.66
4	GO:0051129	GO Biological Processes	negative regulation of cellular component organization	7	13.73	-3.77	-1.48
5	GO:0032868	GO Biological Processes	response to insulin	4	7.84	-3.32	-1.06
6	GO:0001558	GO Biological Processes	regulation of cell growth	5	9.80	-3.14	-0.88
7	M5884	Canonical Pathways	NABA CORE MATRISOME	4	7.84	-2.92	-0.67
8	GO:0008285	GO Biological Processes	negative regulation of cell population proliferation	6	11.76	-2.68	-0.47
9	GO:0010821	GO Biological Processes	regulation of mitochondrion organization	3	5.88	-2.68	-0.47
10	GO:0048469	GO Biological Processes	cell maturation	3	5.88	-2.54	-0.36
11	GO:0071478	GO Biological Processes	cellular response to radiation	3	5.88	-2.45	-0.28
12	WP4352	WikiPathways	Ciliary landscape	3	5.88	-2.24	-0.11
13	GO:0006364	GO Biological Processes	rRNA processing	3	5.88	-2.20	-0.08
14	GO:0021537	GO Biological Processes	telencephalon development	3	5.88	-2.04	0.00

“Count” is the number of genes in the user-provided list with membership in the given ontology term. “%” is the percentage of all of the user-provided genes that are found in the given ontology term. “Log10(P)” is the p-value in log base 10. “Log10(q)” is the multi-test adjusted p-value in log base 10.

Table 5 - Enrichment analysis of biological processes for 68 down-regulated proteins of CMT identified by LC/MS-MS.

Biological Process*	Identified proteins involved in process	Logp
Systemic lupus erythematosus	H2AC8, H2AC7, H2AX, H3-3A, H3-4, H2AC13, H2AC14, H2AC16, H2AC6, H2AC18, H2AC20, H3C1, H3C6, H3C12, H2AJ, H2AC12, H2AC25, H3C14, H2AC1, H3C15, H3-5	-3,59594E+16
FBXL10 enhancement of MAP/ERK signaling in diffuse large B-cell lymphoma	H2AX, H3-3A, H3C1, H3C6, H3C12, H2AJ, H3C14, H3C15	-1,52978E+16
Protein refolding	HSPA1A, HSPA1L	-4,99158E+15
Negative regulation of cellular component organization	APC, FLNA, H3-3A, HSPA1A, PICK1, KIF14	-3,77203E+15
Response to insulin	APC, PKLR, PKM, VPS13C	-3,32442E+15
Regulation of cell growth	H3-3A, HSPA1A, URI1, KIF14, H3-5	-3,14036E+15
Naba core matrisome	COL6A3, DCN, PCOLCE, PRELP	-2,91885E+15
Negative regulation of cell population proliferation	APC, H2AC8, HSPA1A, H2AC6, GNA13, GTPBP4	-2,67981E+16

The Metascape was applied to functionally annotate enriched proteins, using the ontology sources. All genes in the genome have been used as the enrichment background. Terms with a p-value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 (the enrichment factor is the ratio between the observed counts and the counts expected by chance) are collected and grouped into clusters based on their membership similarities. More specifically, p-values are calculated based on the cumulative hypergeometric distribution, and q-values are calculated using the Benjamini-Hochberg procedure to account for multiple testings. Kappa scores are used as the similarity metric when performing hierarchical clustering on the enriched terms, and sub-trees with a similarity of > 0.3 are considered a cluster. The most statistically significant term within a cluster is chosen to represent the cluster

3.3.3 Functional aspects of differentially expressed proteins

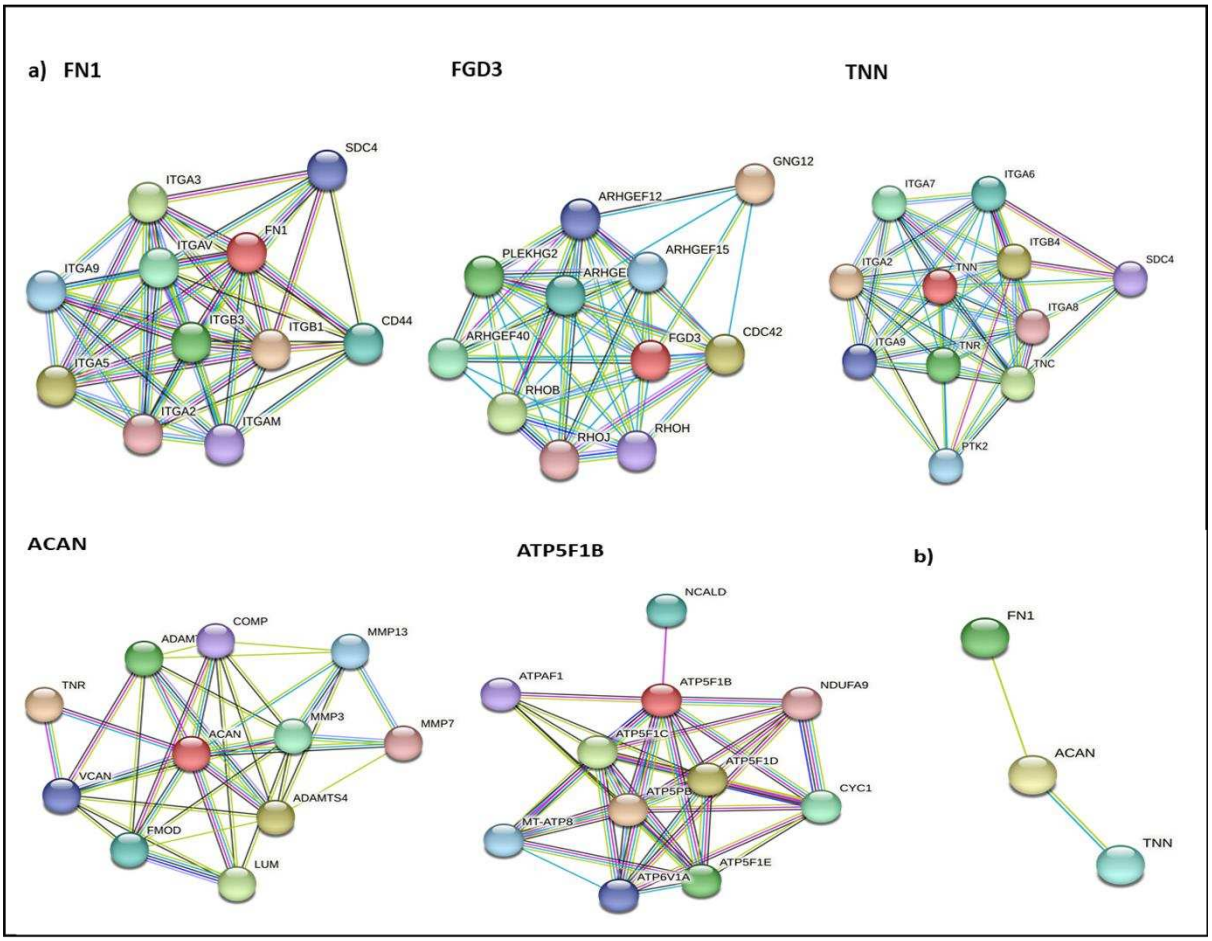
We compared protein abundance between CMT and NT tissues and considering the fold-change values, Fibronectin (FN1), FYVE_ RhoGEF and PH domain protein 3 (FGD3), Tenascin (TNN), Aggrecan (ACAN), and mitochondrial ATP synthase beta_ subunit (ATP5F1B) showed the highest relative abundances in CMT, and FN1 was the most abundant protein among the up-regulated proteins. Concerning the down-regulated proteins in CMT, fold-change values were higher in Tudor domain-containing protein 7 (TDRD7), RNA-binding motif_ single-stranded-interacting protein 1 (RBMS1), and beta-lactoglobulin-1 (LGB1), among others, and TDRD7 was the most abundant protein among the down-regulated proteins (Table 6).

Protein-protein interaction network analysis (Figure 6) was determined by *in silico* models, the most significant components of FN1 interactome included Integrins (ITGB1, ITGA5, ITGA3, ITGB3, ITGAV, ITGA9, ITGAM, ITGA2 (score ≥ 0.998)), CD44 antigen (CD44, score 0.986) and Syndecan-4 (SND4, score 0.966). The array of FGD3 interactions with Guanine nucleotide-binding protein subunit gamma (GNG12, score 0.930), Cell division control protein 42 homologs (CDC42, score 0,914), and Ras homolog family member B (RHOB, score 0,908). In addition, Tenascin N (TNN) shows interaction with Integrin subunit alpha (ITGA2, score 0.918). Interesting interactions of ACAN showed has been involved with Tenascin R (TNR, score 0.969), ADAM metallopeptidase with thrombospondin type 1 motif 4 (ADAMTS4, score 0,923), and Lumican (LUM, score 0,914). Finally, ATP synthase subunit beta_ mitochondrial (ATP5F1B) appear to be connecting nodes with six proteins belonging to ATPases (ATP5PB, ATP5F1D, ATP5F1C, MT-ATP8, ATP6V1A, ATPAF1, (score ≥ 0.999)), Cytochrome c1 (CYC1, score 0,989), Neurocalcin delta (NCALD, score 0,987), and NADH: ubiquinone oxidoreductase subunit A9 (NDUFA9, score 0,966). To find connections between these proteins *in silico* analysis using the to String platform indicated that FN1, ACAN, and TNN shared pathways, domains, and physical interactions.

Table 6 - Proteins in CMT and NT with the highest different abundances.

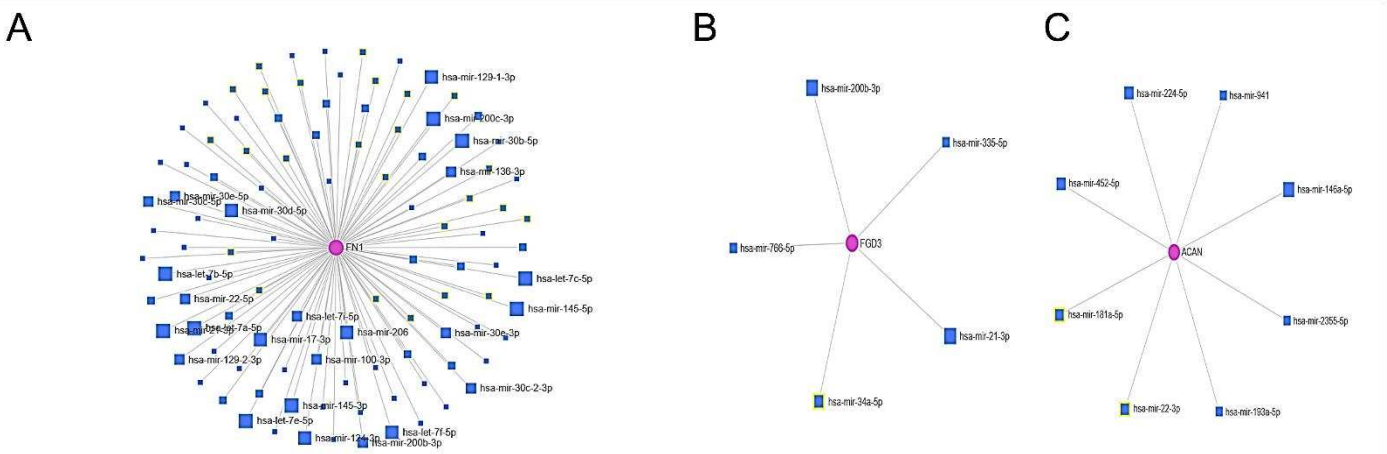
Accesion	Protein description	Higher abundance in	Max fold change	Confidance score	Anova (p)
Q28275	Fibronectin (Fragment) GN=FN1	CMT	153.996731084293	85.9887	0.00340283588371937
Q5JSP0	FYVE_ RhoGEF and PH domain-containing protein 3 GN=FGD3	CMT	55.6202133704164	13.6065	0.0124616809916367
Q9UQP3	Tenascin-N GN=TNN	CMT	45.1786360566808	9.4348	0.00175570390697655
Q28343	Aggrecan core protein GN=ACAN	CMT	22.6473867088306	37.8878	9.79548667543462E-05
Q5ZLC5	ATP synthase subunit beta_ mitochondrial GN=ATP5F1B	CMT	22.45266411967	89.6876	0.00685513032627633
P03994	Hyaluronan and proteoglycan link protein 1 GN=Hapln1	CMT	9.49362224373983	5.3468	0.000160537020974405
O57535	Nucleoside diphosphate kinase GN=NEM1	CMT	8.11737900900414	10.947	1.22411443537196E-07
A5A6N4	Eukaryotic initiation factor 4A-I GN=EIF4A1	CMT	8.10154738106723	9.8406	9.33896976973436E-06
Q8K1H1	Tudor domain-containing protein 7 GN=Tdrd7	NT	644.207404375262	21.4937	0.0731716075308138
Q5PQP1	RNA-binding motif_ single-stranded-interacting protein 1 GN=Rbms1	NT	195.221236753456	12.8929	0.00537539985991609
P33685	Beta-lactoglobulin-1 GN=LGB1	NT	48.5043910734017	211.2833	0.000546497259558021
O77788	Neurofilament medium polypeptide GN=NEFM	NT	10.9084010157999	51.6108	0.0113181230431844
P01977	Hemoglobin subunit alpha-1 GN=HBA1	NT	7.48262770876741	12.8318	0.0573262344157828
P33686	Beta-lactoglobulin-2 GN=LGB2	NT	10.8712636408058	121.258	0.00488410159664354
Q27975	Heat shock 70 kDa protein 1A GN=HSPA1A	NT	5.68875237481207	64.0741	0.0293824486525754

Figure 7 - a) Individual in silico networks associated with FN1, FGD3, TNN, ACAN, and ATP5F1B. b) Combined in silico networks associated between the up-regulated proteins in CMT according to String version 11.5 default settings (https://string-db), using *Canis lupus familiaris* NCBI database.



Based on the analysis conducted with the miRNet 2.0 database, FN1 is regulated by miRNAs associated with cell death, aging, cell proliferation, epithelial-to-mesenchymal transition, apoptosis, and innate immunity. FGD3 gene was regulated by miRNAs associated with cell migration, cell cycle, cell proliferation, and vascular remodeling. Moreover, ACAN is regulated by onco-miRNAs and other miRNAs associated with DNA damage response, pluripotent stem cells reprogramming, and chondrocyte development (Figure 7, Appendix-A).

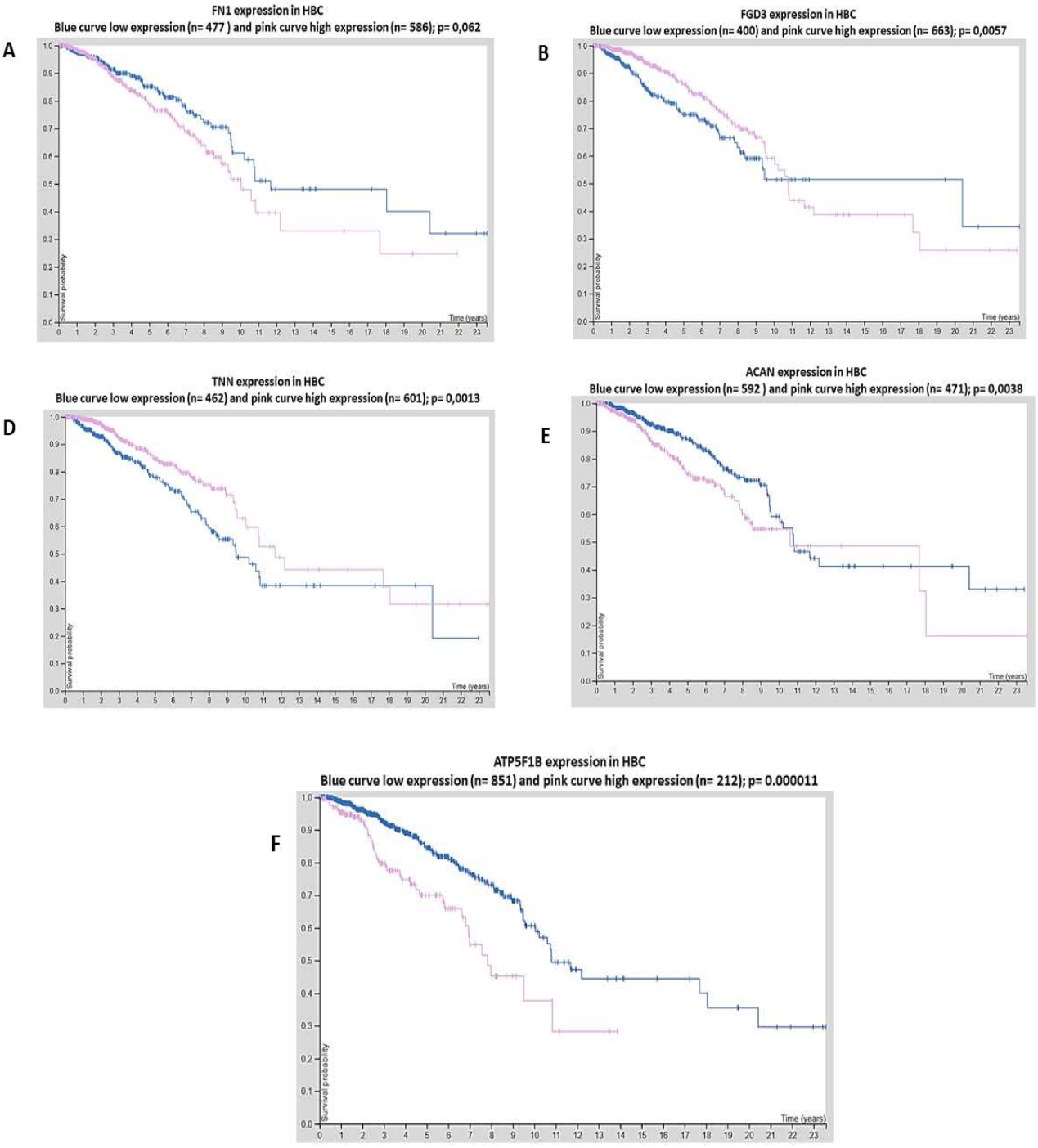
Figure 8 - Network and gene set enrichment analysis of miRNAs associated with the regulation of FN1, FGD3 and ACAN according to the highest p-values obtained from miRNet using *Homo sapiens* database (*https://www.mirnet.ca*).



3.3.4 Prognostic Value of FN1, FGD3, TNN, ACAN, and ATP5F1B in Human Breast Cancer Patients

As demonstrated by functional enrichment analyses, Fibronectin (FN1), FYVE_ RhoGEF and PH domain-containing protein 3 (FGD3), Tenascin-N (TNN), Aggrecan core protein (ACAN), and ATP synthase subunit beta_ mitochondrial (ATP5F1B) were related to the cell proliferation and extracellular matrix (ECM) remodeling in CMT, and thus being promising candidates for further validation as comparative prognostic biomarkers. However, we intended to study the relationship between the gene expression of such proteins and the clinical outcomes of HBC patients using The Human Protein Atlas online database. *In silico* validation explored the OS prognostic value that was obtained according to the low and high expression of each gene. We observed that the expression of FN1 (p score = 0,062), FGD3 (p score = 0,0057), TNN (p score = 0,0013), and ACAN (p score = 0,0038) were not associated with OS in breast cancer patients. However, we observed that the high expression ATP5F1B (p score = 0,000011) is associated with a worse OS in breast cancer patients. As a result, ATP5F1B is prognostic and its high expression is unfavorable in HBC (Figure 8).

Figure 9 - Prognostic values of FN1, FGD3, TNN, ACAN, and ATP5F1B for OS in human breast cancer patients. Gene expression and clinical outcome data were analyzed using using The Human Protein Atlas. The Kaplan–Meier curves indicate a blue curve for a low expression and a pink curve for a high expression.



3.4 Discussion

According to a recent report, breast cancer is one of the most prevalent and deadly cancers among women worldwide (Sung *et al.*, 2021). In the last years, numerous researches have successfully applied proteomic technologies of breast tumor tissues in order to discover biomarkers/protein signatures that are suitable for: early diagnosis, tumor characterization and subtyping; and prognosis and prediction of therapy outcomes (Gromov; Moreira; Gromova, 2014).

Canines with spontaneous mammary tumors have been considered as a useful model for comparative oncology research due to the epidemiological, biological and pathological similarity with HBC, but is greatly understudied (LIU *et al.*, 2014; RIVERA *et al.*, 2009; PINHO *et al.*, 2012). Some proliferation markers, hormone receptors, and oncogenes have been investigated in CMT as potential prognostic biomarkers, but none have been used in veterinary medicine routine. This is partly due to the lack of studies that have demonstrated the prognostic value of these biomarkers (Santos *et al.*, 2013). In the present study, we performed LC-MS to demonstrate a comparison of proteomic profiles of CMT and NT tissues. The enrichment factors related to the up-regulated proteins in CMT would be a strong indication for discrimination between non-tumor tissue.

A total of 945 proteins were identified for all tissues analyzed. The largest number of the proteins were up-regulated (136 proteins) in the CMT tissues when compared to the NT. With respect to identification of proteins that play promoting roles in CMT, this study was centered on up-regulated proteins; however, it is noteworthy that 68 proteins were identified as down-regulated and share important pathways related to cell proliferation and tumor growth.

The results of the CMT tissues used in this study reflect the mixture of all cells present in the sample, not discriminating between mammary tumor cells and other non-tumor cells. It is well known that bulk tumors are a conglomerate of tumor cells with their microenvironment including for instance inflammatory cells, cancer-associated fibroblasts, immune cells, vascular cells, adipocytes, and ECM molecules, which are closely associated with tumorigenesis, cell proliferation, survival, invasion, and metastasis (Markkanen, 2019). In fact, the biological processes related to the up-regulated proteins in CMT in this study showed an involvement with pathways characterizes of microenvironment cells. Hence, a number of significant terms in the pathway enrichment analysis representing an important connection between the tumor microenvironment and tumor cells.

We demonstrated that ALDOA and member of Tubulins, HSP90, HSP70, and 14-3-3 families were differentially up-regulated in CMT and their presence was not restricted to one biological process. Protein folding process occur in the endoplasmic reticulum (ERet) and requires the coordinated activity of many folding chaperones, such as HSP70 and HSP90 (Gardner; Fenger; London, 2016). HSP70 binds to exposed stretches of hydrophobic residues of immature polypeptide chains, while HSP90 facilitates maturation of client proteins to a biologically active conformation. Many of the HSP90 client proteins are oncoproteins such as HER2, EGFR, kinases, among others (Gardner; Fenger; London, 2016). In fact, tumor cells proliferate in an environment that combines nutrient deprivation and dysregulation of protein synthesis, often leading to ERet stress. During ERet stress, its processing is reduced and in response to this stress, both normal cells and tumor cells express various proteins of the protective unfolded protein response, which reduce cell survival by inducing apoptosis (Clarke *et al.*, 2014). In dogs, it is known that there is up-regulation of HSP70 and HSP90 proteins in metastatic CMT and their metastasis sites (Proteome of metastatic canine mammary carcinomas: Similarities to and differences from human breast cancerKlopfleisch, Robert *et al.*, 2010). Interestingly, in our study HSP90AA1 is up-regulated in CMT and according to The Human Protein Atlas, HSP90AA1 is prognostic and its higher expression is unfavorable in HBC strengthening the dog as a valuable comparative model.

As mentioned, HSP90 is a molecular chaperone involved in the activation of numerous client proteins, among them many kinases. The function of HSP90 is to stabilize and mature the kinase toward its active state, but when faced with a state of stress in the unfolding of proteins in the ERet, the kinases eventually destabilize and with that the action of HSP90 is inhibited (Boczek *et al.*, 2015). Furthermore, we observed that the 14-3-3 protein family was associated with the maturation Kinase 1 complex as well as with the translocation process of SLC2A4 (GLUT4) to plasma membrane processes, which occurs in adipocytes and myocytes. The latter process emphasizes the important interaction between tumor cells and non-tumor cells. In a study using invasive human breast cancer cell lines, it showed that HSP90AB1 and YWHAB (member of the 14-3-3 family) were highly expressed, with YWHAB being involved in activating tumor progression and metastasis pathways, as well as inhibiting apoptosis in HBC (Tilli *et al.*, 2016). According to The Human Protein Atlas, YWHAB is prognostic and its higher expression is unfavorable in HBC. Interestingly, in our study, HSP90AB1 and YWAB were also up-regulated in CMT, reinforcing important implications for comparative oncology research.

The beta tubulins are part of the microtubules and perform multiple important cellular functions such as structural support, pathway for transport, and force generation in cell division (Binarová; Tuszynski, 2019). Therefore, they are essential for cell division and growth in normal cells. However, the up-regulation of specific tubulin isotypes is associated with energy metabolism, immune system activation, and drug resistance in cancer cells (Binarová; Tuszynski, 2019). It is known that energy metabolism of cancer cells even in the presence of oxygen can reprogram their glucose metabolism, and thus their energy production, limiting their energy metabolism largely to glycolysis, leading to a state that has been called "aerobic glycolysis" (WARBURG, 1956). Although the mitochondrial oxidative phosphorylation process of normal cells is more efficient than glycolysis, the energy generation presented by tumor cells favors the faster production of the energy needed to supply cell growth and proliferation (Hanahan; Weinberg, 2011). This phenomenon may explain the high rates of glucose uptake observed in most tumors.

Neutrophils are the first line of innate immunity to respond to tissue injury inflammation and capture invading microorganisms through different mechanisms such as phagocytosis, degranulation, and formation of neutrophil extracellular traps. However, in humans, neutrophils exhibit phenotypic heterogeneity and functional versatility and may contribute to tumor progression, playing multiple mechanisms such as promotion of angiogenesis, immunosuppression, and cancer metastasis (WU *et al.*, 2020).

Our study demonstrated that the upregulation of ALDOA, HSP90AA1, YWHAE, and TUBB8 was related to VEGFA-VEGFR2 signaling pathways. Like normal tissues, tumors have nutrient and oxygen needs. As tumors grow, they exceed the capture of these molecules in the existing vascular network, as well as the ability to evacuate metabolic waste products. Thus, the tumor creates a new vascular network that supplies these needs during a process called angiogenesis (Hanahan; Weinberg, 2011). One of the most important factors contributing to angiogenesis is vascular endothelial growth factor (VEGFA). VEGFA interacts with the transmembrane receptor tyrosine kinase VEGFR, which is expressed on vascular endothelial cells, and this interaction is the main pathway that activates angiogenesis (Simons, 2016). These observations mentioned before may reflect the impacts of these proteins on tumor development and progression in a canine model of breast cancer.

We compared protein abundance between CMT and NT tissues, and FN1, FGD3, TNN, ACAN, and ATP5F1B had the greatest abundances in the CMT. Among this select group, FN1 showed the highest abundance. FN1 is a high molecular weight adhesive

glycoprotein, which is abundant in the ECM and body fluids, participating in various processes involved in cancer cell progression by interacting in the crosstalk between cells and ECM molecules (HAN; LU, 2017). Similar to the FN1, ACAN is a proteoglycan, and the high expression in certain types of CMT as complex adenomas and mixed tumors, suggests a cartilaginous potential of ACAN in these tumors (ERDÉLYI *et al.*, 2005). In fact, reflects in our analysis that the majority of the CMT included two benign mixed tumors and two complex adenomas. TNN is another ECM protein highly expressed in the stroma of most solid tumors. A previous study states that TNN is part of the niche for HBC metastasis sites, supporting cell migration and tumor proliferation (DEGEN *et al.*, 2007). A further study reported the presence of TNN in a large number of low-grade breast tumors and promoted the migration of HBC cells to fibronectin (CHIOVARO *et al.*, 2015). The presence of a specific type of tenascin as the tenascin-C (TNC) was abundant in CMT, mainly in areas of initial chondroid metaplasia and in cartilage islands in mixed tumors (FAUSTINO *et al.*, 2002). However, to our knowledge, this is the first report to suggest that TNN may also play an important role in CMT.

Our analysis showed that ATP5F1B was abundant among CMT members. ATP5F1B is the major catalytic subunit of ATP synthase, involved in energy metabolism (Xu *et al.*, 2009). Furthermore, ATP5F1B is the most stable gene in canine mammary tissues (Etschmann *et al.*, 2006) and has been used as a housekeeping gene in primary sequences in several studies (da Costa *et al.*, 2011, 2012; Klopfleisch; Gruber, 2009; Klopfleisch; Klose; Gruber, 2010; Proteome of metastatic canine mammary carcinomas: Similarities to and differences from human breast cancerKlopfleisch, Robert *et al.*, 2010; Lee *et al.*, 2018). In HBC, ATP5F1B has been shown to be a key player involved in tumor progression and a higher risk of metastasis, and its high expression is associated with worse OS (Liu *et al.*, 2021). Accordingly, in our study, the high expression of the ATP5F1B gene was associated with worse OS, therefore, ATP5F1B had a significant prognostic value for OS in human breast cancer patients. In contrast, a higher abundance of FGD3 seems to indicate a lower risk of metastasis by inhibiting cell migration. According to RENDA *et al.* (2019), FGD3 is a significant independent prognostic factor in ≤ 40-years-old women suffering from breast cancer in terms of DFS)and OS. In another study, an elevated expression of FGD3 showed significantly better DFS and OS in women with diagnostic of invasive breast cancer (Susini *et al.*, 2021). However, to date, no study has demonstrated the presence of FGD3 in CMT.

Among the proteins that contribute to the interactive network described in our study, we found that FN1 linked to the Integrins, CD44 and SDC4. Like FN1, TNN also shows

a connection with an Integrin. Integrins are related to cell adhesion and play a crucial role in regulating the various stages of metastasis and determining the aggressiveness of cancer cells (Gkretsi; Stylianopoulos, 2018). Syndecans show an affinity for many ECM proteins helping to bind cells to each other. In addition, syndecans are also showing involvement in both tumor suppression and proliferation progression in various types of human cancers, including HBC (Czarnowski, 2021). CD44 is a cell surface antigen that is used in human medicine to identify CSCs. Immunohistochemical analyses on CSCs in both CMT and HBC show that CD44 has an influence on prognosis, with the CD44+/CD24- phenotype being related to a poor prognosis (Im *et al.*, 2015; Kaszak *et al.*, 2018; Magalhães *et al.*, 2013). In addition, the FGD3 assembly showed binding to RHOB, as well as a binding to CDC42. RHOB and CDC42 are known to participate in cell-ECM adhesions and are associated with higher metastatic potential or lower OS in several types of human cancers (Gkretsi; Stylianopoulos, 2018). In HBC, higher levels of these components have been found in late stage tumors and metastases with prognostic relevance (Fritz *et al.*, 2002). Combined *in silico* analysis based on the String platform showed associations between FN1, ACAN, and TNN, indicating that the actual work of these ECM proteins certainly involves more complex mechanisms than expected when only their individual roles are analyzed.

In silico models also identified that onco-miRNAs and miRNAs linked to DNA damage response, pluripotent stem cells reprogramming, and chondrocyte development are capable of regulating ACAN expression. MiR-146a-5p has been reported to be significantly overexpressed in HBC promoting cell proliferation, while down expression of miR-146a-5p may inhibit cell proliferation. In addition, BRCA1 was further identified as a target gene of miR-146a-5p expression acting in the regulation of cell proliferation (Gao *et al.*, 2018). A study suggests that miR-22-3p functions as a tumor suppressor and that loss of miR-22-3p contributes to the growth and progression of triple-negative breast cancer, which has a high aggressiveness and insensitivity to chemotherapy (Gorur *et al.*, 2021).

Fibronectin is regulated by miRNAs that are associated with cell proliferation and epithelial transition, such as miR-124-3p, whose decreased expression promoted breast cancer proliferation and metastasis (Yan *et al.*, 2019). Moreover, down-regulation of miR-124 in canine mammary carcinoma was associated with histological quality and metastasis (Ren *et al.*, 2022). FN1 is also regulated by miR-155-5p which was related to aging, innate immunity, and apoptosis. MiR-155-5p stands out as a prominent oncomiR and its high expression has

been associated with high-grade tumors, advanced stages, and lymph node metastasis, as well as worse DFS and OS in breast cancer patients (Pasculli *et al.*, 2020).

FN1 and FGD3 genes were regulated by miR-21-3p in various pathways, including cell death, aging, innate immunity, and cell proliferation. In HBC, elevated levels of miR-21-3p were associated with lymph node positivity, as well as an association with pathological characteristics that suggested a worse prognosis. Specifically, overexpression of miR-21-3p was associated with large tumors, a high grade, lymph node involvement, HER2 positivity, and shorter breast cancer-specific survival (Amirfallah *et al.*, 2021). In addition, FN1 and FGD3 were regulated by miR-200b-3p and miR-200c-3p. Downregulation of miR-200b-3p and miR-200c-3p were detected in inflammatory breast cancer, which is a rare and aggressive breast cancer variant, associated with a poor prognosis (Fahim *et al.*, 2020).

3.5 Conclusion

This study highlighted the molecular landscapes of CMT to HBC using methods of proteomics and bioinformatic tools. We showed that FN1, FGD3, TNN, ACAN, and ATP5F1B may be key factors involved in tumorigenesis in the canine model of breast cancer, and ATP5F1B would also have significant prognostic value as demonstrated in survival analysis based on the HBC gene database. However, more experiments are needed to validate FGD3 and TNN expression as well as their prognostic significance in CMT samples. Thus, further comparative studies can be applied to canine mammary tumors and many other types of cancer, given their close similarity with human disease.

4 FINAL CONSIDERATIONS

In conclusion, the biological processes of the analyzed CMT were shown to be related to the pathobiology of the disease, as were the proteins involved in the majority of biological enrichments. Among the most abundant proteins in CMT, the identification of ECM-related proteins was evident. Also, connections were observed between the most abundant proteins with further proteins that are involved in tumorigenesis, tumor progression, and metastasis. Finally, it was identified for the first time in CMT the presence of FGD3 and TNN, which leads us to conclude that the expression of these proteins already identified in HBC may also perform the same function in CMT.

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APPENDIX A - BIOLOGICAL ENRICHMENT ANALYSIS OF MIRNAS ASSOCIATED WITH THE REGULATION OF FN1, FGD3, AND ACAN.

	CELL DEATH	AGING	EPITHELIAL-TO-MESENCHYMAL TRANSITION	CELL PROLIFERATION	INNATE IMMUNITY	APOPTOSIS
FN1	hsa-let-7a-5p	hsa-let-7g-5p	hsa-mir-26b-5p	hsa-mir-200b-3p	hsa-let-7g-5p	hsa-let-7g-5p
	hsa-let-7b-5p	hsa-mir-200c-3p	hsa-mir-200b-3p	hsa-let-7g-5p	hsa-let-7a-5p	hsa-mir-1-3p
	hsa-let-7c-5p	hsa-let-7a-5p	hsa-mir-200c-3p	hsa-mir-200c-3p	hsa-let-7b-5p	hsa-mir-125a-3p
	hsa-let-7e-5p	hsa-let-7b-5p	hsa-let-7a-5p	hsa-mir-140-3p	hsa-let-7c-5p	hsa-mir-130a-3p
	hsa-let-7f-5p	hsa-let-7c-5p	hsa-let-7b-5p	hsa-let-7a-5p	hsa-let-7e-5p	hsa-mir-132-3p
	hsa-let-7i-5p	hsa-let-7e-5p	hsa-let-7c-5p	hsa-let-7b-5p	hsa-let-7f-5p	hsa-mir-16-5p
	hsa-mir-128-3p	hsa-mir-100-3p	hsa-let-7e-5p	hsa-let-7c-5p	hsa-mir-136-3p	hsa-mir-181a-2-3p
	hsa-mir-130b-3p	hsa-mir-145-5p	hsa-mir-100-3p	hsa-let-7e-5p	hsa-mir-145-3p	hsa-mir-193b-3p
	hsa-mir-145-5p	hsa-mir-151a-5p	hsa-mir-124-3p	hsa-mir-124-3p	hsa-mir-145-5p	hsa-mir-19b-3p
	hsa-mir-16-5p	hsa-mir-16-5p	hsa-mir-145-5p	hsa-mir-130a-3p	hsa-mir-181a-2-3p	hsa-mir-204-3p

	hsa-mir-17-3p	hsa-mir-17-3p	hsa-mir-29b-3p	hsa-mir-145-5p	hsa-mir-21-3p	hsa-mir-210-3p
	hsa-mir-19b-3p	hsa-mir-21-3p	hsa-mir-30a-3p	hsa-mir-16-5p	hsa-mir-24-3p	hsa-mir-222-5p
	hsa-mir-21-3p	hsa-mir-26a-5p	hsa-mir-30a-5p	hsa-mir-17-3p	hsa-mir-26a-5p	hsa-mir-24-3p
	hsa-mir-24-3p	hsa-mir-30a-3p	hsa-mir-30b-5p	hsa-mir-199a-5p	hsa-mir-29b-3p	hsa-mir-26a-5p
	hsa-mir-29b-3p	hsa-mir-30a-5p	hsa-mir-30c-2-3p	hsa-mir-21-3p	hsa-mir-30a-3p	hsa-mir-29b-3p
	hsa-mir-30b-5p	hsa-mir-30b-5p	hsa-mir-30c-5p	hsa-mir-224-5p	hsa-mir-30a-5p	hsa-mir-30a-3p
	hsa-mir-30c-2-3p	hsa-mir-30d-5p	hsa-mir-30d-5p	hsa-mir-24-3p	hsa-mir-30b-5p	hsa-mir-30a-5p
	hsa-mir-30c-5p	hsa-mir-30e-3p	hsa-mir-30e-3p	hsa-mir-29b-3p	hsa-mir-155-5p	hsa-mir-34a-5p
	hsa-mir-30d-5p	hsa-mir-30e-5p	hsa-mir-30e-5p	hsa-mir-34a-5p		hsa-mir-34b-5p
	hsa-mir-34a-5p	hsa-mir-34a-5p	hsa-mir-34a-5p			hsa-mir-424-5p
	hsa-mir-7-5p	hsa-mir-7-5p				hsa-mir-497-5p
	hsa-mir-98-5p	hsa-mir-155-5p				hsa-mir-7-5p

	hsa-mir-23b-3p					hsa-mir-92a-2-5p
						hsa-mir-155-5p
						hsa-mir-449b-5p

	CELL MIGRATION	**CELL CYCLE**	**CELL PROLIFERATION**	**VASCULAR REMODELING**
FGD3	hsa-mir-34a-5p	hsa-mir-21-3p	hsa-mir-21-3p	hsa-mir-34a-5p
	hsa-mir-200b-3p	hsa-mir-34a-5p	hsa-mir-34a-5p	
		hsa-mir-200b-3p	hsa-mir-200b-3p	
	ONCO-MIRNAs	**DNA DAMAGE RESPONSE**	**PLURIPOTENT STEM CELLS REPROGRAMMING**	**CHONDROCYTE DEVELOPMENT**
ACAN	hsa-mir-22-3p	hsa-mir-452-5p	hsa-mir-181a-5p	hsa-mir-181a-5p

	hsa-mir-224-5p	hsa-mir-146a-5p	hsa-mir-22-3p	hsa-mir-146a-5p
	hsa-mir-146a-5p			