UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

IMPROVING FELINE MAMMARY CARCINOMA TREATMENT THROUGH HER2-RELATED IMMUNOCHEMOTHERAPY AGENTS AND BIOMARKERS

Andreia Pereira Gameiro

Orientador(es): Professor Doutor Fernando António da Costa Ferreira

Professor Doutor Jorge Manuel de Jesus Correia

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade Ciências Biológicas e Biomédicas.

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Ano de conclusão (indicar o da data da realização das provas públicas): 2022

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Para os meus pais,

AGRADECIMENTOS

É verdade que sozinhos nunca conseguiremos nada, e é neste espirito de entreajuda e apoio que me foi possível concluir mais uma etapa importante na minha formação. Foram várias as pessoas que me acompanharam ao longo deste percurso, a quem não posso deixar de agradecer.

Assim, deixo o meu muito obrigado ao meu orientador, Professor Fernando Ferreira, pelo convite que me fez à 4 anos atrás, para a realização deste projeto e sempre me incentivou com novas ideias e objetivos, ao longo desta jornada. Agradeço também ao meu coorientador, Professor Jorge Correia, pelo apoio e ensinamentos que me foi transmitindo, no que se refere à anatomia patológica e ainda, à Doutora Maria João Soares, que com muito esforço pessoal contribuiu para a existência de um excelente banco de tumores, os quais foram utilizados em parte dos estudos científicos que realizei. Por último, nesta "equipa", quero agradecer aos colegas que me acompanharam no dia-a-dia de experiências, alegrias e aborrecimentos. À Catarina Nascimento, ao Filipe Almeida, à Cláudia Marques, ao Ferdinando Freitas e à Sandra Carvalho, agradeço pela companhia e apoio, pelos ensinamentos que me foram passando e, sobretudo por me aturarem no dia-a-dia. Quero deixar também um agradecimento aos colegas do almoço e aos colegas de laboratório, às várias equipas da Faculdade de Medicina Veterinária, com as quais me fui cruzando ao longo destes anos e às minhas colegas do Mestrado Integrado em Medicina Veterinária, que mesmo em conversas banais, me foram apoiando e incentivando ao longo deste percurso, acabando todos eles por, de alguma forma, se tornarem importantes.

Por fim agradeço aos meus pais e irmão, que contribuíram em tudo para o que sou hoje e me ensinaram sempre a lutar pelo que quero, não importa o esforço necessário, com eles aprendi que no final, compensa. Ao meu namorado, pois sei que estes anos não têm sido fáceis, muito trabalho, muita pressão, horários fora do normal. Por isso, obrigada por toda a paciência, por estares ao meu lado e me aturares no dia-a-dia.

Por fim obrigada à FCT e ao CIISA pela possibilidade de realização deste trabalho e ainda ao HEV – FMV, ULisboa, ao IMM, Fundação Champalimaud e ao IPO – Lisboa, pois de uma forma ou de outra contribuíram para o sucesso final deste projeto de doutoramento.

Consegui assim chegar ao final desta caminhada, com todos os objetivos cumpridos. Obrigada a todos, por de alguma forma fazerem parte deste percurso e da minha vida pessoal, foi com um pouquinho de cada um que cheguei até aqui.

FINANCIAL SUPPORT

This research was funded by:

- Ph.D. fellowship from Fundação para a Ciência e a Tecnologia, Portugal SFRH/BD/132260/2017.
- PTDC/CVT-EPI/3638/2014: “Molecular evaluation of HER2 and topoisomerases in feline mammary carcinoma – Developing rational strategies for effective diagnosis and cancer chemoimmunotherapy” from Fundação para a Ciência e a Tecnologia.
- Project UIDB/00276/2020 (CIISA – Centro de Investigação Interdisciplinar em Sanidade Animal).

MELHORAR O TRATAMENTO DO CARCINOMA MAMÁRIO FELINO ATRAVÉS DE IMUNOQUIMIOTERAPIA DIRIGIDA PARA O HER2 E NOVOS BIOMARCADORES TUMORAIS

RESUMO

O carcinoma mamário felino (CMF) é um tumor frequente, sendo os subtipos HER2-positivo e triplo-negativo os mais agressivos, tal como na mulher. No gato, este tumor é geralmente diagnosticado num estadio avançado, havendo pouca informação sobre o seu desenvolvimento e opções terapêuticas limitadas, para além da mastectomia, o que resulta num curto tempo de sobrevivência. Assim, este projeto foi desenvolvido de forma a melhorar as opções terapêuticas, na gata, bem como identificar novos biomarcadores tumorais, relacionados com a proteína HER2 e tirando vantagem dos protocolos validados em medicina humana. De forma a encontrar diferentes opções terapêuticas para o tumor de mama, diversos fármacos (TKi – lapatinib e neratinib; mAbs – trastuzumab e pertuzumab; ADC – T-DM1; e inibidor da via mTOR – rapamicina, como adjuvante) e protocolos de conjugação, foram testados usando linhas celulares imortalizadas de CMF (CAT-M, FMCp e FMCm). Estes ensaios exibiram efeitos antiproliferativos promissores, apresentando mecanismos de ação conservados, induzindo baixos níveis de fosforilação das proteínas alvo, aquando do uso de TKi e, apoptose, quando os mAbs e ADC foram testados. Em paralelo, os protocolos de conjugação apresentaram excelentes respostas sinérgicas. Ainda, amostras de tecido de tumores de mama de gata foram analisadas, por forma a identificar mutações no her2, como indicadores de prognóstico, ou resistência à terapêutica. Os resultados obtidos revelaram uma mutação, no exão 18 (c.19573 A>T), associada a tumores de maiores dimensões, um fator de mau prognóstico. Além disso, nenhuma mutação caracterizada se encontra descrita na mulher, como responsável pela resistência terapêutica. Adicionalmente, considerando a correlação entre obesidade, com aumento dos níveis de leptina e o desenvolvimento do tumor de mama HER2-positivo, na mulher e, uma vez que na gata este é um problema nutricional comum, o eixo leptina/recetor (ObR) foi avaliado, como possível biomarcador tumoral. Os resultados obtidos mostraram que gatos com carcinoma mamário apresentam uma diminuição do índice de leptina livre, estando os níveis elevados de leptina associados a mau prognóstico e, os níveis séricos de ObR relacionados com um estado de imunossupressão geral. Em conclusão, os resultados obtidos suportam o uso de fármacos anti-HER2 e ainda, o uso da leptina e do seu recetor como biomarcadores tumorais e possíveis alvos adjuvantes à terapia.

Palavras-chave: Carcinoma mamário felino; HER2; mutações no her2; biomarcadores tumorais; terapias dirigidas

IMPROVING FELINE MAMMARY CARCINOMA TREATMENT THROUGH HER2-RELATED IMMUNOCHEMOTHERAPY AGENTS AND BIOMARKERS

ABSTRACT

The feline mammary carcinoma (FMC) is a common tumour, with the HER2-positive and triple-negative being the most aggressive subtypes, as in women. This tumour is usually diagnosed belatedly, with scarce information about its development, and limited therapeutic options beyond mastectomy, presenting the cat a low overall survival time. Thus, this project was developed in order to improve therapeutic options, as well as, to reveal new tumour diagnosis/prognosis biomarkers, related to HER2 protein and taking advantage for the extensive knowledge in protocols used in humans. In order to disclose different therapeutic options for diseased cats, several drugs (TKi – lapatinib and neratinib; mAbs – trastuzumab and pertuzumab; ADC – T-DM1; and mTOR inhibitor – rapamycin, as adjuvant) and combined protocols were tested using FMC cell lines (CAT-M, FMCp and FMCm). These assays revealed promising antiproliferative effects and conserved molecular responses, inducing lower phosphorylation levels of target proteins, when TKi were used, and apoptosis, when mAbs and ADC drugs were evaluated. In parallel, combined protocols presented excellent synergistic responses. Moreover, feline tumour clinical tissue samples were analysed in order to identify *her2* mutations as prognosis markers or therapy resistance indicators. The obtained results correlates a *her2* mutation, in exon 18 (c.19573 A>T), to larger tumour sizes, a poor prognosis feature. Furthermore, any of the mutations found are described, in woman, as inducing therapeutic resistance. Additionally, considering the close relationship between obesity with increased leptin levels and the development of HER2-positive breast cancer, in woman, and since in cat obesity is a frequent nutritional disorder, the leptin/leptin receptor (ObR) axis was evaluated as possible tumour biomarker. Interestingly, cats with mammary carcinoma presented a decreased free leptin index, being the higher leptin levels associated to poor prognostic features, and serum ObR levels were correlated to an immunosuppressive status. In conclusion, the results obtained support the use of therapeutic drugs targeting the HER2, in order to improve cats' prognosis, which could contribute for an advance in the veterinary oncology practice. Furthermore, leptin and ObR were suggested as tumour biomarkers, being proposed its use as putative adjuvant therapeutic targets.

Keywords: Feline mammary carcinoma; HER2; *her2* mutations; tumour biomarkers; targeted therapies

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3D	Tri-dimensional
5-AzaC	5-Azacytidine
ADC	Antibody-drug conjugate
ADCC	Antibody-dependent cellular cytotoxicity
ASCO	American Society of Clinical Oncology
ATP	Adenosine triphosphate
AUC	Area under the curve
BSA	Bovine serum albumin
Ck	Cytokeratin
CKAP5	Cytoskeleton-associated protein 5
COSMIC	Catalogue of Somatic Mutations in Cancer
COX-2	Cyclo-oxygenase 2
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
CXCR4	C-X-C chemokine receptor type 4
CXCL12	C-X-C Motif Chemokine Ligand 12
DFS	Disease free survival
DM-1	Maytansine
DMEM	Dulbecco's modified eagle medium
DMSO	Dymetilsulfoxide
ECD	Extracellular domain
EE	Elston and Ellis Grading System
EGFR	Epidermal growth factor receptor family
ER	Oestrogen receptor
FBS	Fetal bovine serum
FFPE	Formalin fixed paraffin-embedded
FLI	Free leptin index
FMC	Feline mammary carcinoma
GSK-3	Glycogen synthase kinase 3
HBC	Human breast cancer
HDACi	Histone deacetylase inhibitor
HER2	Human epidermal growth factor receptor 2
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
ICD	Intracellular domain
IHC	Immunohistochemistry

LAG-3	Lymphocyte activation gene 3 protein
Lys	Lysine
mAb	Monoclonal antibody
MTi	Microtubule inhibitor
mTOR	Mammalian target of rapamycin
mTORC	Mammalian target of rapamycin complex
mTORi	mTOR inhibitor
ObR	Leptin receptor
OS	Overall survival
PAD	Peptidyl arginine deiminase enzymes
PBS	Phosphate buffered saline
PD-1	Programmed cell death-1
PD-L1	Programmed cell death-ligand 1
PI	Propidium iodide
PR	Progesterone receptor
ROC	Receiver-operating curve
RON	Macrophage-stimulating protein receptor
RPMI	Roswell Park Memorial Institute 1640 medium
RT	Room temperature
SNP	Single nucleotide polymorphism
SVs	Single variants
T-DM1	Trastuzumab-emtansine
TK	Tyrosine kinase
TKi	Tyrosine kinase inhibitors
TNF-α	Tumour Necrosis Factor α
TNM	Tumour, Necrosis, Metastasis
TopBP1	Topoisomerase IIβ binding protein 1
VEGF	Vascular endothelial growth factor
VISTA	V-domain immunoglobulin suppressor of T cell activation
WHO	World Health Organization

Chapter I

Introduction and Literature Review

This chapter was adapted from **Emerging Biomarkers and Targeted Therapies in Feline Mammary Carcinoma** (Review)

Andreia Gameiro, Ana Catarina Urbano and Fernando Ferreira (2021), Veterinary Sciences – MDPI, DOI: 10.3390/vetsci8080164

1. Introduction

Cats are the most popular companion animals in developed countries, outnumbering dogs (Murray et al. 2015). Because they share similar environmental conditions with their owners, as well as genetic and biological features, cats have been used as models for human ophthalmic diseases, type 2 diabetes and, since the full sequencing of their genome, in comparative oncology studies (Hoenig et al. 2000; Zappulli et al. 2005; Henson and O'Brien 2006; Menotti-Raymond et al. 2010). Cats are also emerging as promising animal models for preclinical testing of HER2-positive and triple-negative mammary carcinoma therapies (De Maria et al. 2005; Burrai et al. 2010; Wiese et al. 2013; Almeida et al. 2021).

In accordance to World Health Organization (WHO; 2021), despite the progression in cancer treatment, this condition is one of the most common causes of death worldwide, with high incidence in developed countries. Furthermore, WHO, 2021 report the breast cancer as the second cause of death, in women. By comparison, feline mammary carcinoma (FMC) is the third most common type of cancer in cats, corresponding to 17% of all tumours in queens, and is usually malignant (Novosad 2003), as is human breast cancer (HBC) (Panieri 2012), occurring in 90% of the cases due to somatic mutations (Santos et al. 2013) and showing comparable risk factors. It is the first cause of death in cats, with short overall survival (OS), and very poor prognosis, as it tends to be diagnosed at late stages and has limited therapeutic options that show weak responses (Vail and Macewen 2000; Zappulli et al. 2005). FMC has similar anatomical, biological and clinical features to HBC, although metastatic mechanisms remain poorly understood (Zappulli et al. 2005), and is likewise classified in different molecular subtypes: luminal A, luminal B, epidermal growth factor receptor 2 (HER2)-positive and triple-negative normal-like and basal-like (M. Soares, Correia, Peleteiro, et al. 2016; Maria Soares, Madeira, et al. 2016).

Using the extensive knowledge available on HBC, it is possible to find comparable diagnostic and prognostic biomarkers, as well as therapeutic targets, like the HER2 protein, that may improve FMC's prognosis. This epidermal growth factor receptor (EGFR) family member is commonly targeted in breast cancer therapies by antibodies and/or small inhibitors that disrupt different cellular pathways (Cho et al. 2003; Phillips et al. 2008; Canonici et al. 2013; Schroeder et al. 2014; Richard et al. 2016; Cocco et al. 2018; von Minckwitz et al. 2019). Other emerging agents that have already proved valuable in FMC *in vitro* studies (Almeida et al. 2021) include histone deacetylase inhibitors (HDACi) (Huang and Pardee 2000; Munster et al. 2001), and microtubules inhibitors (MTi) (Risinger et al. 2015; Steinmetz and Prota 2018; Zang et al. 2018).

The research conducted for this thesis aimed to discover new diagnostic and prognostic biomarkers, by evaluating diseased animals and tumour samples, as well as putative targets, to improve therapeutic options for cats with mammary carcinoma. For this purpose, and since it is known a close relation between leptin and its receptor with the development of the HER2-positive HBC, this axis was evaluated in diseased queens, by comparison to its human counterpart. Moreover, HBC therapeutic protocols, targeting the HER2 protein and presenting excellent results, improving women OS and disease-free survival (DFS), such as tyrosine kinase inhibitors (TKi; lapatinib and neratinib), monoclonal antibodies (mAbs; trastuzumab and pertuzumab) and antibody-drug conjugate compounds (ADC; trastuzumab-emtansine – T-DM1) were tested in different cell-based FMC models (CAT-M[1], FMCp and FMCm). Furthermore, combined protocols using the above-mentioned drugs and rapamycin, a mammalian target of rapamycin inhibitor (mTORi) were tested, in order to infer about its utility as new therapeutic protocols for cats with mammary carcinomas.

1.1. Feline Mammary Carcinoma

FMC is a common disease in middle-aged to old queens (10 to 12 years) (Weijer et al. 1972; Giménez et al. 2010), more frequent in the Siamese and domestic short-hair breeds, with an OS time of around 1 year (Millanta et al. 2005; Giménez et al. 2010; Maria Soares, Madeira, et al. 2016; M. Soares, Correia, Peleteiro, et al. 2016). It occurs more frequently in unspayed cats, being associated to the expression of oestrogens (ER) and progesterone (PR), and hormonal therapy (Overley et al. 2005). Indeed, an ovariohysterectomy before 6 months of age is known to be a protective factor, reducing FMC development in 91% of cases (Novosad 2003; Overley et al. 2005). Mammary tumours are usually malignant (80 to 90%), occurring with higher frequency in the abdominal glands and in 50% to 90% of the cases leading to metastasis (Giménez et al. 2010), most commonly in the regional lymph nodes and lungs (Novosad 2003) (**Figure 1**). Nowadays, in cats, only age, breed and hormonal influence are identified as tumour risk factors, despite in women other conditions were known, such as obesity, which increases leptin production and induces a chronic inflammatory condition (Bonofiglio et al. 2019).

[1] CAT-M cell line, could also be referred as CAT-MT.

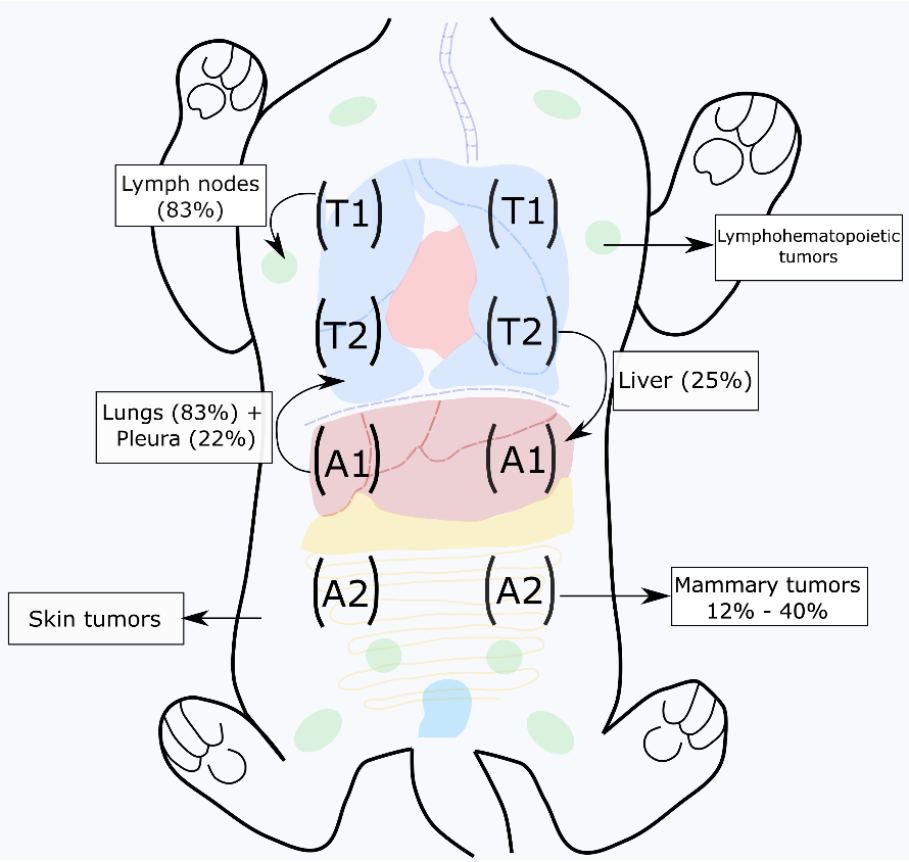

Figure 1. Mammary carcinoma is the third most common tumour in cats, with a high metastasis rate, frequently to lymph nodes and lungs (Giménez et al. 2010; Maria Soares, Madeira, et al. 2016). The black arrows indicate the most frequent tumour locations and metastasis pattern; "A" defines the abdominal and "T" the thoracic mammary glands.

In cats, tumours are easy to detect on the physical exam, appearing as firm and discrete masses in the mammary gland. However, at the time of diagnosis, identification of multiple masses, with higher frequency in the abdominal glands (Weijer and Hart 1983) is common, usually in the same mammary chain, whereas in women a single mass is observed in most cases. The same anatomic classification (*in situ vs.* infiltrative) and histologic grade (Zappulli et al. 2005) are reported for FMC and HBC. Thus, mammary tumours are defined as simple or complex, with secretory and ductal cells documented, and identified as inflammatory disease, when less differentiated cells and lymphatic-dermic obstruction are present.

For veterinaries the recommendation is a complete physical examination, complete blood count, serum biochemical profile, serum T4 concentration, urinalysis, three-view thoracic radiographs, abdominal ultrasound or a computed tomography scan. The mammary masses and palpable regional lymph nodes should be subjected to a fine-needle aspiration or to a biopsy. Ulcerated lesions should also be analysed, in order to have a proper diagnosis before surgery. All the lesions should be analysed by histopathology.

1.1.1. Mammary tumours histologic classification

Early-stage mammary tumours present as mobile, palpable and discrete masses, however, as tumour diagnosis is usually belated, patients tend to present several masses, with ulceration (25% of the cases) and necrosis. The physical exam may also reveal oedema, exudate in the nipples and a decrease in the temperature of the pelvic region. For correct diagnosis and prognosis before surgery, a precise tumour classification is mandatory. Even though cytology is easy to perform, most of the times results are inconclusive (Novosad 2003), making biopsy crucial to confirm tumour stage and malignancy grade (Giménez et al. 2010).

In cats, is known that, more than 85% of the mammary lesions are malignant, occurring in 15% of the cases dysplastic lesions, characterized by ductal hyperplasia, lobular hyperplasia, cysts, duct ectasia and fibrosclerosis, or fibroadenomatous changes hormonal induced, with proliferation of interlobular ducts and periductal stroma cells (Misdorp et al. 1999). Malignant tumours present an aggressive biological behaviour, with lymphatic invasion and lymph node metastasis. These tumours type are usually adenocarcinomas, as the tubulopapillary and solid carcinomas. Other types described could include *in situ* carcinoma, cribiform carcinoma, invasive micropapillary carcinoma, squamous cell carcinoma, mucinous carcinoma, lipid-rich carcinoma and inflammatory mammary carcinoma (Misdorp et al. 1999; Pérez-Alenza et al. 2004; Kamstock et al. 2005; Seixas et al. 2007; Millanta et al. 2012).

Although a standardized classification system does not exist, the same parameters used for HBC are applied in FMC (**Table 1**), with TNM (Tumour, Node and Metastasis) being the most widely used staging system (Preziosi et al. 2002; Chocteau et al. 2019). Tumour classification also considers the malignancy grade, which takes into account tumour size, tissue invasion, tumour ulceration, lymphovascular invasion, and lymph node status (Chocteau et al. 2019). Additionally, a histopathological analysis is recommended, with higher frequencies of adenocarcinomas *in situ*, tubullopapilary, solid, or cribiform masses reported. Concerning histologic grade, the Elston and Ellis (EE) Grading System (Elston, C.W. and Ellis 1998) is usually employed, and as recommend by Mills et al., 2015, it is also important to take into consideration the lymphovascular invasion, nuclear form and mitotic counting. Using this system, the majority of tumours are defined as moderate to less differentiated masses (Giménez et al. 2010; Mills et al. 2015).

Table 1. Tumour clinical stage and histological grade for feline mammary carcinoma (Adapted from the System modified from Owen LN., Classification of tumours in domestic animals, Geneva World Health Organization, 1980; and Elston & Ellis Grading System, 1998, respectively).

Tumour classification of feline mammary carcinomas			
Tumour Clinical Stage*			
Tumour size (T)	**Lymph node status (N)**	**Metastasis (M)**	**Stage**
T1 (<2cm)	N0	M0	1

T2 (2-3cm)	N0	M0	2
T1 / T2	N1	M0	3
T3 (>3cm)	N0 / N1		
Any	N0 / N1	M1	4

Histological Grade (EE System)

Histologic feature	**Score**
Tubule formation	
>75%	1
10 - 75%	2
<10%	3
Nuclear pleomorphism	
Mild	1
Moderate	2
Marked	3
Mitotic count (*per* 10 microscopic fields)	
0-5	1
6 - 10	2
>11	3

Sum of the scores	**Grade**
3-5	I
6-7	II
8-9	III

*0 – indicates absence of the characteristic; 1 – indicates presence of the characteristic.

1.1.2. Molecular classification

Considering the heterogeneity in HBC, and assuming similar features in cat, the molecular characterization, by St. Gallen International Expert Consensus in luminal A, luminal B, HER2-positive and triple-negative subtypes (Maria Soares, Madeira, et al. 2016), reveals itself as an important prognostic factor (Perou et al. 2000), and may unveil targets for a directed therapy. The above-mentioned tumour subtypes were described below, with special emphasis for the HER2-positive and triple-negative tumours, the most prevalent and aggressive subtypes.

Tumours molecular classification are defined by gene expression levels. However, it was proved that immunohistochemistry (IHC), which defines protein expression it is a cheaper and easy to perform technique which reveals the tumour gene expression pattern (Nielsen et al. 2004; Goldhirsch et al. 2013), being able to categorize the tumours and anticipate its prognosis.

1.1.2.1. Luminal A

According to the St. Gallen International Expert Consensus guidelines, luminal A tumour subtype is characterized by the overexpression of ER and/or PR, absence of HER2 and a low Ki-67 index (below 14%), reflecting its low proliferation rate (Perou et al. 2000; Maria Soares, Madeira, et al. 2016).

Clinically, this tumour subtype is characterized by a low histological grade, good prognosis and higher OS times (Perou et al. 2000; Kennecke et al. 2010; Eroles et al. 2012).

Moreover, the luminal A subtype has a distinct metastization pattern, with higher incidence of bone metastases, and lower risk for liver and lung metastasis (Kennecke et al. 2010).

1.1.2.2. Luminal B

Luminal B tumour subtype is characterized by ER- and/or PR-overexpression, and in 30% of the cases could also overexpress EGFR or HER2 proteins (Eroles et al. 2012). Additionally, the Ki-67 index occurs above 14% (M. Soares, Ribeiro, Carvalho, et al. 2016), proving its elevated proliferation rate, being an aggressive tumour subtype, with higher histological grade and lower DFS (Kennecke et al. 2010; Eroles et al. 2012).

In this case, metastasis are more common in bone tissue and liver (Kennecke et al. 2010).

1.1.2.3. HER2-positive

Mammary HER2-positive tumours, in cats, are described with a prevalence of 30% to 60%, being one of the most aggressive subtypes, as in women (Maria Soares, Ribeiro, et al. 2016; Maria Soares, Madeira, et al. 2016).

This subtype occurs with a HER2-overexpression, and usually ER- and/or PR-negative, according to St. Gallen International Expert Consensus guidelines (Goldhirsch et al. 2013; M. Soares, Correia, Peleteiro, et al. 2016). Moreover, cats with HER2-positive tumor subtype presented an immunosuppression status (Nascimento et al. 2020; Urbano et al. 2020; Nascimento et al. 2021), as described in human patients.

Clinically, as in HBC, it is observed a high proliferation rate (Ki-67 index > 14%) (M. Soares, Ribeiro, Carvalho, et al. 2016) and tumour grade, being associated to a poor prognosis, with shorter DFS and OS rates (Maria Soares, Ribeiro, et al. 2016). In addition, metastasis are more common in regional lymph nodes, bone tissue and lungs (Novosad 2003).

1.1.2.4. Triple-negative

Finally, the triple-negative subtype is described as being diagnosed from 37% to 54% of the FMC cases (Millanta et al. 2006; Maniscalco et al. 2013). The triple-negative tumour is characterized by the absence of hormonal receptors (ER and PR) and HER2 expression (Nielsen et al. 2004), being usually identified a high expression of the EGFR protein (Badve et al. 2011).

In general, triple-negative tumours are aggressive, with a high metastization rate, in visceral organs, as lungs, central nervous system and lymph nodes (Rakha et al. 2009; Eroles et al. 2012). Furthermore, they could be classified with high histologic tumour grade, large tumour sizes, marked cellular pleomorphism, high nuclear-cytoplasmic ratio, lack of tubule formation, high mitotic index (Ki-67 index > 14%), frequent apoptotic cells, tumour necrosis and an evident stromal lymphocytic response (Rakha et al. 2009; Bosch et al. 2010).

Clinically, this subtype as a huge diversity of presentations, being described as triple-negative basal-like, and normal-like if negative for basal biomarkers, such as cytokeratin (Ck) 5/6 and EGFR. In cats, the most aggressive subtype was identified as the triple-negative normal-like (Nascimento et al. 2020; Nascimento et al. 2021). However, for the purpose of this study, basal- and normal-like triple-negative tumours were considered as a whole and defined as triple-negative subtype.

1.2. Prognostic factors

In cats, prognosis is evaluated using the one-year postsurgical survival or remission rate, comparable to the ten-year postsurgical survival or remission rate, in humans. By comparison to the HBC knowledge, several are the prognostic indicators that could be considered for the FMC. Furthermore, to uncover new diagnostic and prognostic biomarkers, as well as new therapeutic targets, the study of tumour microenvironment, its molecular characterization, and the analysis of systemic alterations on diseased animals, becomes crucial.

The FMC is a very aggressive neoplasia, being reported a metastization rate of 50% to 93%, identified at necropsy (Weijer and Hart 1983; Hahn and Adams 1997; Giménez et al. 2010). For cats, the best prognostic indicators are the DFS and post-surgical remission rate (MacEwen et al. 1984; Matos et al. 2012), with a survival time after tumour diagnosis, dependent on clinical stage and surgical procedure, being around 12 months (Zappulli et al. 2005). In the physical examination several parameters become important to evaluate, since they can condition the prognosis, e.g. age, presenting the old animals lower survival rates (Weijer and Hart 1983; Ito et al. 1996). In addition, considering the tumour macroscopic analysis, size is one of the most important prognostic factors in FMC, with masses larger than 3 cm presenting a poor prognosis (Mills et al. 2015), and conditioning a more aggressive surgical approach (McNeill et al. 2009).

Furthermore, the tumour's histologic grade, presence of lymphatic metastasis and/or lymphovascular invasion (Novosad 2003), as well as tumour stage (Ito et al. 1996) and subtype (Mills et al. 2015) are described as highly correlated with OS. Thus, lymph node metastasis were associated to shorter OS (Seixas et al. 2011) and the non-metastatic tumours treated with radical mastectomy presenting higher DFS and OS rates (Weijer and Hart 1983; MacEwen et al. 1984). Deepening tumour characterization, clinical stage is also an important prognostic feature. Thus, tumour classification based on TNM system stage III and IV are associated to lower OS and DFS rates (Castagnaro et al. 1998; Seixas et al. 2011; Hughes and Dobson 2012). Furthermore, low hormonal dependency, such as triple-negative subtype is associated to higher malignancy grade and more aggressive behaviours (Martín De Las Mulas et al. 2002; Millanta et al. 2005; Millanta et al. 2006; Burrai et al. 2010). In parallel, high mitotic rate and

the proliferative biomarker Ki-67, were associated to low OS and DFS (Preziosi et al. 2002; Novosad 2003; Millanta et al. 2006; Seixas et al. 2011; M. Soares, Ribeiro, Carvalho, et al. 2016).

1.2.1. Tumour biomarkers as valuable prognostic factors

Despite the identification of tumour biomarkers in cats are a relatively recent field of study, several proteins have already been identified, as may be involved in FMC prognosis. Molecular expression of Ki-67, reveals that an index above 14% is associated with poor prognosis (M. Soares, Ribeiro, Carvalho, et al. 2016). AKT expression, which is usually associated with PR-/ER-negative invasive carcinomas, and correlated with malignancy and non-tumour differentiation, lowering the DFS (Maniscalco et al. 2012). In parallel, cats with a triple-negative subtype present increased mammalian target of rapamycin (mTOR) expression, as has been described in women (Watanabe et al. 2011), being associated with cancer invasion and metastasis (Maniscalco et al. 2013). Moreover, mutations in the *p53* gene involved in cell cycle regulation and tumour suppression have been reported in 18.9% of FMC (Murakami et al. 2000; Nakano et al. 2006).

Furthermore, the overexpression of several molecular biomarkers are also associated with poor prognosis, e.g. macrophage-stimulating protein receptor (RON), related to tumour invasion, cyclo-oxygenase (COX)-2, expressed in malignant FMC, and topoisomerase IIβ binding protein 1 (TopBP1), which is similar to BRAC2 in HBC (Morris et al. 2008; Giménez et al. 2010). Interestingly, the C-X-C chemokine receptor type 4 (CXCR4)/ C-X-C motif chemokine ligand 12 (CXCL12) axis, which controls cell survival, migration and proliferation, is also a key factor in feline breast cancer progression and metastasis, as reported for HBC (Müller et al. 2001; Marques et al. 2018), and it's disruption is associated with lower OS time (F. Liu et al. 2009; Marques et al. 2017; Marques et al. 2018). Additionally, CXCL12 has been reported as a blood serum marker in cat, particularly for HER2-positive tumours (Marques et al. 2017). Finally, analysis of the vascular endothelial growth factor (VEGF) status, shows that this molecule is overexpressed in more aggressive carcinomas (McNeill et al. 2009; Nascimento et al. 2021), playing an important role in tumour-associated angiogenesis.

In parallel, an association is reported between the expression of some biomarkers in serum and tumour microenvironment, suggesting that serum samples may be used as a non-invasive method for the assessment of checkpoint proteins (Marques et al. 2017; Nascimento et al. 2020; Papadaki et al. 2020). Interestingly, in cat, HER2 serum expression increases in malignant lesions, lowering OS (Giménez et al. 2010; Maria Soares, Madeira, et al. 2016), making it a promising diagnostic tool. In fact, our group has already shown that a rapid diagnosis kit for the identification of HER2-positive mammary carcinoma, through the detection of serum HER2 expression levels can be produced. These preliminary experiment, using

nanoparticles coated with anti-HER2 fluorescent antibodies, showed that serum HER2 expression levels can be quantified in cats with mammary carcinoma (**Figure 2A**), by comparison with a control sample (**Figure 2B**). Nevertheless, more work is needed in order to define cut-off values, sensitivity and specificity of the test.

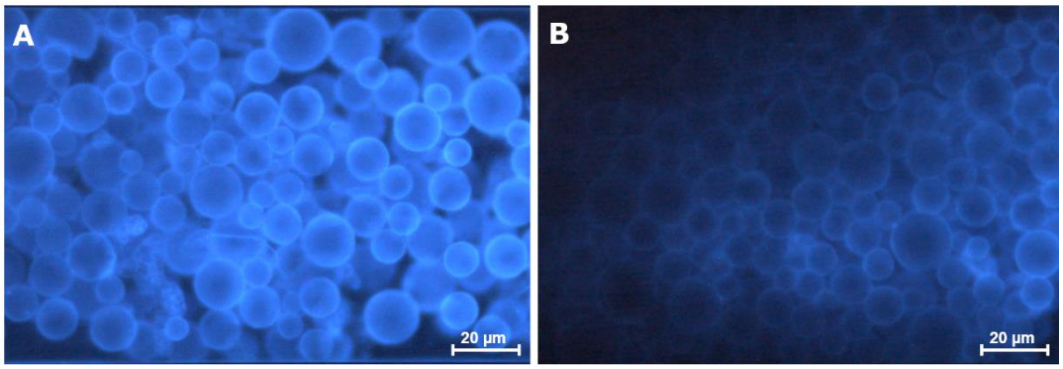

Figure 2. Serum HER2 protein levels measured using nanoparticles coated with an anti-HER2 antibody. **A)** The nanoparticles were coated with a fluorescent anti-HER2 antibody (CB11), allowing quantification of serum HER2 expression levels in cats with HER2-positive mammary carcinoma (3+ score), by comparison with a **B)** control serum sample from a healthy animal. This experiment corresponds to preliminary results from a recent study (data not published; 400x magnification).

Still, it is widely acknowledged that in women, a chronic inflammatory status, such as that induced by obesity, can be a trigger for mammary tumour development (Hosney et al. 2017; Pan et al. 2018). Interestingly, in cats obesity is a common nutritional disorder, making the leptin/leptin receptor axis an interesting new putative tumour biomarker.

1.3. HER2 and breast cancer development

The *her2* is an oncogene, located at chromosome E1, in cat (Muscatello et al. 2019), and by comparison, in human is located at chromosome 17 (Jiang et al. 2012). This gene encodes for HER2, a 185 kDa protein, which belongs to the EGFR family (**Figure 3**) (Rampaul et al. 2002; Witton et al. 2003; Ferguson 2008).

EGFR is a family of transmembrane glycoproteins, composed by a N-terminal extracellular domain (ECD), a transmembrane helix domain and an intracellular domain (ICD), presenting tyrosine kinase (TK) activity and a regulatory region (Yarden 2001; Witton et al. 2003; Ferguson 2008). In addition, the ECD is composed by four subdomains, two leucine-rich locations (I and III), responsible for the ligand interaction among the EGFR family members, and two cysteine-rich locations (II and IV), with disulphide-bond activity for ligand-induced dimerization (Ferguson 2008; Sun et al. 2015). This family is composed by four homologous receptors (HER1, HER2, HER3 and HER4), which interact with ligands in its ECD. Specifically, the HER2 protein allows homo or heterodimerization, by the EGFR members (Rampaul et al. 2002), being activated by phosphorylation of its TK domain (Witton et al. 2003; Ferguson 2008; Park et al. 2008; Sun et al. 2015), and also presenting a basal TK activity (Citri and Yarden

2006). In parallel, HER3 is the exception of this family members, presenting no kinase activity (Lyu et al. 2018).

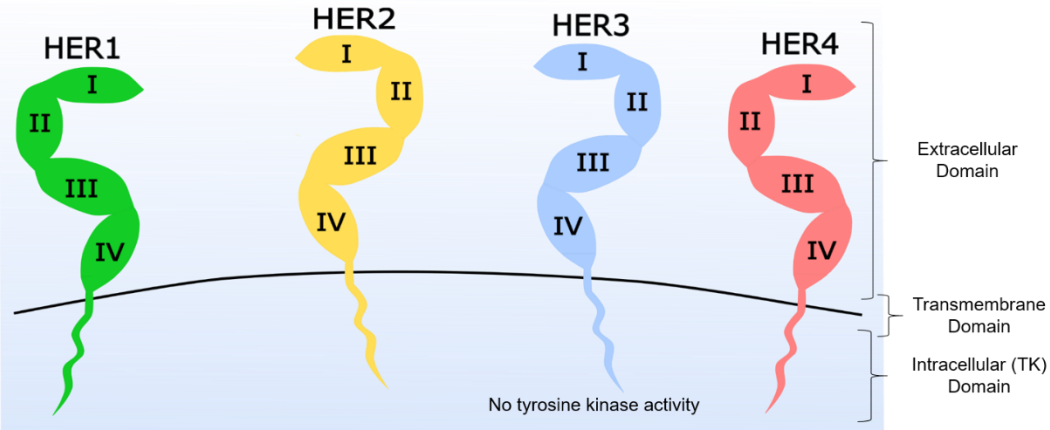

Figure 3. Schematic representation of the EGFR family members (HER1, HER2, HER3 and HER4).

1.3.1. HER2 pathways and molecular development of breast cancer in human

In human, HER2 plays a critical role in the mid-gestation process, being present in the development of cardiovascular and nervous systems and mammary gland. Additionally, in adults, HER2 is crucial for the heart function, being expressed mainly in the cardiomyocytes (Ménard et al. 2004). In parallel, in the mammary gland, all the EGFR family members are involved in cell growth, differentiation, apoptosis and remodelation of the normal tissue. The four receptors are differentially expressed in the mammary gland development, with HER1 and HER2 being crucial in the major cell types at the initial development, and HER3 and HER4 preferentially expressed in the lactating ducts and alveoli (Schroeder and Lee 1998).

HER2 is the preferred ligand-induced dimerization partner, from all the EGFR members (Garret et al. 2003). The activation of the EGFR receptors leads to a complex biological network (**Figure 4**), starting at the ECD, with signal-processing, which leads to the activation of the TK domain, and culminates in the generation of an output signal, activating downstream pathways (Benusiglio 2007). Thus, depending on the ligand composition and the ligand-receptor pair involved, different cell pathways could be activated, with different magnitude and duration (Marmor et al. 2004; Hynes and MacDonald 2009), e.g. RAS-ERK pathway, involved in cell proliferation and the PI3K-AKT-mTOR pathway, which controls cell apoptosis (Frogne et al. 2009).

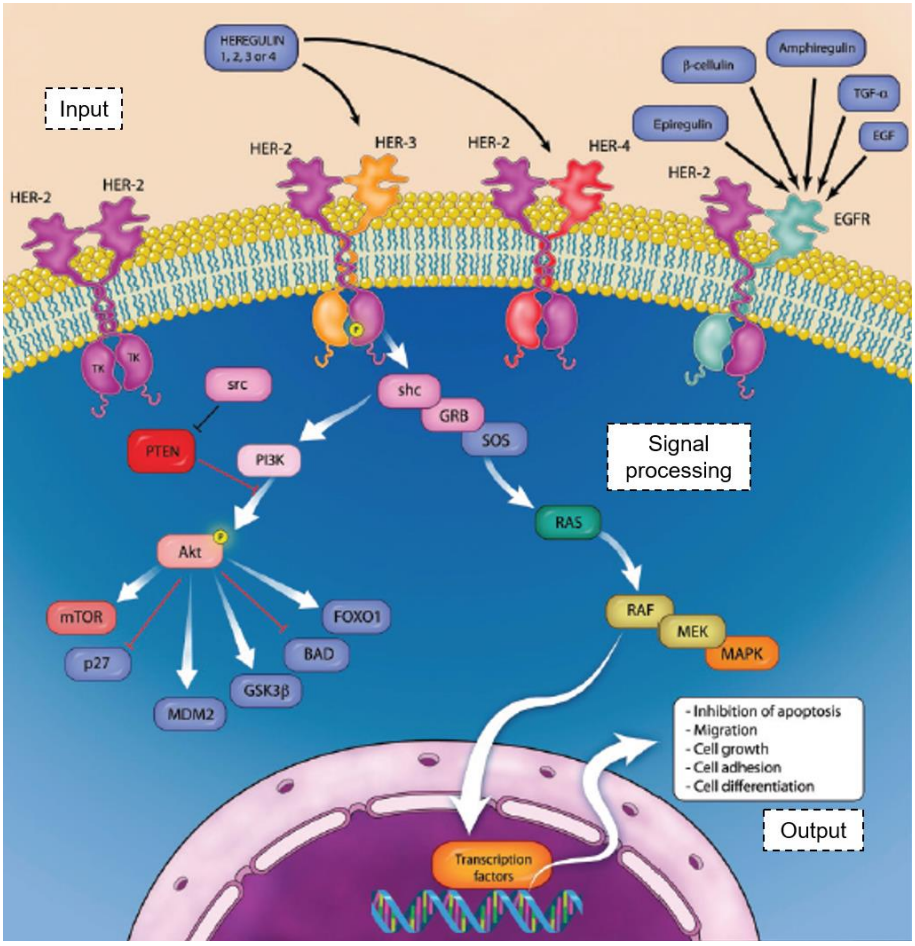

Figure 4. HER2 induces the activation of a complex pathway. In the input layer different ligands and dimeric receptor combinations are represented; then the signal is processed, by the phosphorylation of tyrosine kinase residues, culminating in the activation of different cell pathways; the output signal is involved in cell cycle progression and inhibition of apoptosis. Adapted from Okines & Cunningham, 2012.

The relation among HER2 and HBC development is widely studied. Is known that dysregulations in the HER2 network could induce neoplastic properties to cells, as proliferation, migration, angiogenesis, stromal invasion and resistance to apoptosis (Marmor et al. 2004).

Analysing tumour clinical samples, *her2* mutations are described as more common in HER2-negative tumours. Its majority are characterized as missense mutations in both, ECD and ICD or duplications/insertions, near the exon 20 (Arteaga and Engelman 2014; Zabransky et al. 2015). Not only these mutants, but also *her2* polymorphisms increase the HER2 signalling and autophosphorylation inducing cellular transformations (Benusiglio et al. 2005). In parallel, the amplification of the *her2* gene occurs in 10 to 40% of breast cancer, being correlated with the increase in the mRNA, and leading to the overexpression of HER2. This dysregulation is responsible for the formation of HER2-positive tumours, with an aggressive behaviour and poor prognosis (Meng et al. 2004; Jiang et al. 2012; Vicario et al. 2015).

Furthermore, the overexpression of HER2 activates AKT, a protein that presents a central role in the tumorigenesis process (Holbro et al. 2003; Moasser 2007). Additionally,

HER2 overexpression, induces cell proliferation by disrupting the regulation of cell cycle progression and apoptosis (Frogne et al. 2009), and promotes metastization, inducing the secretion of basement membrane degradative enzymes, as the metalloproteinases (Ménard et al. 2003). Also, HER2 shedding by proteolytic cleavage, in the juxtamembrane region of the protein, caused by zinc-containing metalloproteases members (Sanderson et al. 2006; Tsé et al. 2012), produces truncated forms of the protein that are able of kinase activity. As a result of this shedding, soluble HER2 fragments composed by the ECD, could be quantified in serum (Tsé et al. 2012), and described as a marker of poor prognosis (Carney et al. 2013).

1.3.2. Feline HER2

In cats, *her2* is located in chromosome E1, being codified by 28 exons (*felis catus*, GenBank: NC_018736.2). The transcript is defined by 3738 base pairs (*felis catus*, GenBank: AY702651) and the final translated protein is composed by 1260 aminoacids (*felis catus*, GenBank: AAW23986.1), presenting 90% to 95% identity, with its human counterpart (Zappulli et al. 2005; Santos et al. 2012). As in humans, the feline HER2 is composed by an ECD, a transmembrane region and an ICD with TK activity (De Maria et al. 2005; Santos et al. 2013).

Interestingly, if on one hand HBC progression could be associated with HER2 overexpression, due to an increase in *her2* gene copy numbers (Meng et al. 2004; Jiang et al. 2012; Vicario et al. 2015), on the other hand, a different process was described in cat. FMC development was associated to an increase in *her2* mRNA copy numbers (Soares et al. 2013; Ferreira, Soares, et al. 2019; Muscatello et al. 2019), with some studies suggesting this amplification as a protective factor (Santos et al. 2013).

Similarly to what is described in HBC, in cat, 90% of the breast tumours have acquired somatic mutations (Santos et al. 2013), mostly occurring in the TK domain (Santos et al. 2013; Muscatello et al. 2019), with two single variants (SV) and two haplotypes described (Santos et al. 2012). Considering the *her2* gene sequence that encodes for the HER2 ECD, three non-synonymous genomic variants were reported, predicting an alteration of the 3D (three-dimensional) structure of the protein (Santos et al. 2013). Furthermore, the gene expression analysis suggests the presence of feline *her2* gene post-transcriptional regulation, truncated forms and single-point mutations in the FMC (Santos et al. 2013). The observed *her2* mutations are suggestive of an association with the clinicopathological features, being correlated with primary tumour size and the number of tumour masses (Santos et al. 2012). Furthermore, SVs at splicing regions, *her2* polymorphisms, or mutations in introns may be originating different isoforms of the protein, leading to an increase in HER2 activity and tumour aggressiveness (Santos et al. 2012) or therapy resistance (Rockberg et al. 2009; Kanthala et al. 2016).

1.4. The use of in vitro models and the cat as a comparative oncology model

For the approval of any therapeutic drug, different experimental systems are considered, starting with cell lines, animal models and finally clinical trials. Thus, comparative oncology studies arises with a major importance, being the cat already proved as a valuable model (Zappulli et al. 2005; Cannon 2015), allowing to unveil new knowledge into cancers' biology and therapy (Paoloni and Khanna 2008).

Cell lines revealed to be valuable *in vitro* models for the screen of new therapeutic agents that could be used in mammary carcinomas treatment. These *in vitro* assays are the first line of research and allow to predict cancer cells cytotoxic response, assuming a high degree of genomic similarity (Kao et al. 2009; Wei et al. 2015), but they not represent the full tumour microenvironment, neither a proper systemic response. Considering the cat as an *in vitro* model, such studies are limited, however, by a lack of feline cell lines available for cytotoxicity assays, with only eight cell lines having been reported so far (Uyama et al. 2005).

After the initial *in vitro* research, the next step consider *in vivo* models, in order to understand the real systemic effect and effectiveness of the therapeutic protocols. Thus, new drugs for breast cancer treatments have been investigated mainly in murine cancer models, which represents an unquestionable important line of research, but they have some limitations (Young 2008; Wei et al. 2015). Unlike humans, cats or dogs, in murine models, tumours have to be induced, which conditions biological differences that can lead to divergences in the carcinogenesis process (Rangarajan and Weinberg 2003). Moreover, some limitations have to be considered, e.g. murine's physiology, such as the limitation in emesis, a poor immune system and differences in pharmacodynamics and pharmacokinetic of the drugs. Still, concerning the study of FMC in these models, the options remains scarce, with only four xenograft models recently reported (Chuang et al. 2021). Thus, the use of companion animals arise in order to surpass some of these limitations and to contribute for more viable clinical trials, a better consent of the public and to preserve the animal rights (Vail and Macewen 2000).

Considering the cat as an *in vivo* model, it shares the same environment with his owners and several biological and molecular characteristics, being valuable for pharmacokinetics and toxicology studies. Furthermore, advantages are known when drugs' doses are extrapolated to humans, with special importance for the phase I clinical trials, allowing to decrease its duration, and reducing the associated morbidity and mortality (de las Mulas and Reymundo 2000; Porrello et al. 2006; Paoloni and Khanna 2008; Airley 2012). Other important advantages of the use of these animals as models are its size, which makes easier the sample collection process and allows more viable studies of cell/tumour interactions, disease progression and immunological response (Vail and Macewen 2000; Khanna and Hunter 2005; Airley 2012).

1.5. Treatment options for cats with mammary carcinoma, by comparison to human breast cancer therapeutic protocols

In cats diagnosed with mammary carcinoma, therapeutic options are scarce, the most common being uni/bilateral radical mastectomy, alone, or in combination with chemotherapeutic adjuvant protocols when the Ki-67 index is above 14% (M. Soares, Ribeiro, Carvalho, et al. 2016), which increase cat's DFS, but not OS, due to the high metastization rate (Novosad 2003; Giménez et al. 2010; Michishita et al. 2016). Moreover, the agents used tend to have limited efficacy and severe side effects (Vail and Macewen 2000; Zappulli et al. 2005). Combined therapeutic protocols, using doxorubicin and cyclophosphamide/carboplatin, for example, presented poor response in metastasis (Jeglum et al. 1985; Giménez et al. 2010), and tamoxifen shows no significant response (Zappulli et al. 2005), as FMC is more commonly ER-negative, unlike HBC (Overley et al. 2005; Zappulli et al. 2005).

Thus, a deep understanding is needed to unveil alternative therapeutic options aimed at improving the cat's clinical outcome. Resorting to the extensive knowledge provided by HBC therapeutic protocols, several options could be tested, targeting the HER2 (**Figure 5**), in FMC cell lines, as alternative for the treatment of diseased cats.

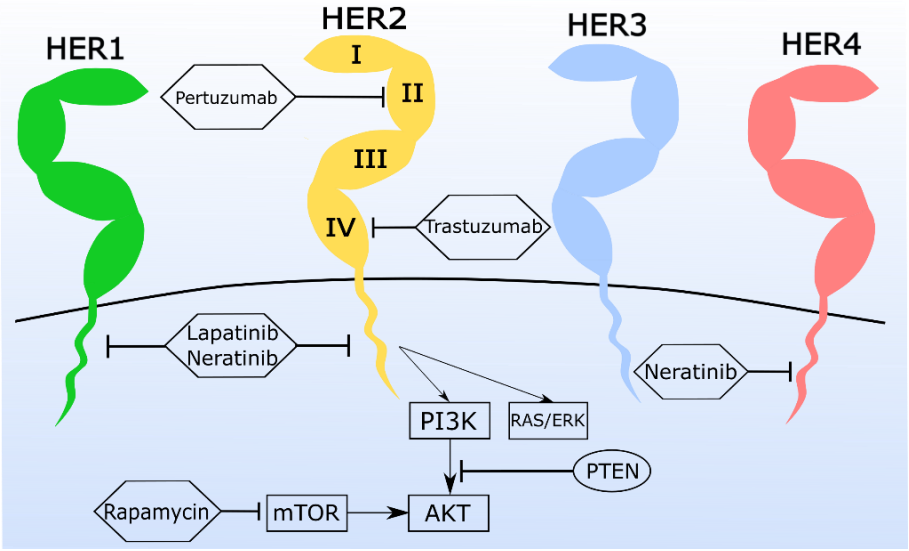

Figure 5. The HER2 pathway is a good candidate for a targeted therapy, in cats with mammary carcinoma, using different classes of compounds, proved valuable in human breast cancer therapeutic protocols.

1.5.1. Small molecule inhibitors

TKis are small chemical compounds that prevent protein phosphorylation, by interacting with the cytoplasmic catalytic kinase domain of different proteins (Schroeder et al. 2014), e.g. EGFR family members. These compounds block the HER2 signalling for cell proliferation via the RAS-ERK pathway (Matkar et al. 2017) and cell death inhibition via the PI3K-AKT-mTOR pathway (Faber et al. 2009). Despite the known side effects, they are a

suitable alternative for patients that show resistance to anti-HER2 mAbs, reported as around 50%, in women with HER2-positive breast cancer (O'Brien et al. 2010; Schroeder et al. 2014).

Different TKis are approved in market, and as example, lapatinib is an oral reversible EGFR inhibitor, competing with the adenosine triphosphate (ATP)-binding pocket of HER1 and HER2 proteins (Shi et al. 2016). This compound suppresses HER2 downstream pathways, namely MAPK-ERK1/2 and PI3K-AKT, leading to apoptosis (Schroeder et al. 2014). In parallel, neratinib is an irreversible pan-HER inhibitor, presenting a similar mechanism of action. This molecule that interacts with HER1, HER2 and HER4 receptors (Tiwari et al. 2015), has more efficacy reported in HER2-positive breast cancers. Furthermore, neratinib reveals to be important in lapatinib- and/or trastuzumab-resistant patients (Canonici et al. 2013; Segovia-Mendoza et al. 2015).

1.5.2. Monoclonal antibodies

The HER2 protein is a common target for molecular therapy in HBC patients, using mAbs that interact by shape complementarity (Cho et al. 2003), thus preventing HER2 dimerization and activation of its downstream pathways (Yamashita-Kashima et al. 2017). These compounds are a good alternative to TKi, which are toxic for the majority of tissues, showing severe side effects (Bonkobara 2015). Recent studies have revealed a 93% similarity between human and feline HER2 (De Maria et al. 2005; Santos et al. 2013) (*homo sapiens*, UniProt P04626; and *felis catus,* UniProt H9BB15), which allowed for testing of humanized-mAbs against FMC cells. These antibodies are specie-specific, being produced by fusion of the antigen-binding region with the framework region of the specie-specific IgG, minimizing the immunogenicity (Hudis 2007), when *in vivo* tests are performed.

Common mAbs used in HBC therapeutic protocols, are as example, trastuzumab a monoclonal IgG antibody that inhibits the HER2 activation (Richard et al. 2016), and promotes cell apoptosis (Hudis 2007; Valabrega et al. 2007). In parallel, pertuzumab is a recombinant IgG1 antibody anti-HER2 (Mullen et al. 2007; Oh and Bang 2020), mostly valuable in combined therapeutic protocols (von Minckwitz et al. 2017). This mAb inhibits the HER2 pathway, leading to cell-cycle arrest and apoptosis (Yamashita-Kashima et al. 2017).

1.5.2.1. Antibody-drug conjugate compounds

ADCs are molecules composed by an antibody covalently conjugated, using a linker, with a chemical compound. These drugs were developed in order to have a precise delivery of highly potent cytotoxic agents to specific cell targets, decreasing its side effects (Lambert and Chari 2014).

Considering the HER2-positive tumour subtype, the ADC trastuzumab-emtansine (T-DM1) is currently in use in HBC therapeutic protocols (Lambert and Chari 2014). This drug is composed by a trastuzumab antibody linked to a derivative of maytansine (DM1), a potent

microtubule polymerization inhibitor (Barok et al. 2011; Barok et al. 2014). In this way, T-DM1 includes all the mechanisms of action and advantages of trastuzumab, as well as the benefits of DM1 improving DFS and OS rates, and decreasing the toxicity induced by the DM1 molecule (Verma et al. 2012).

1.5.3. Mammalian target of rapamycin (mTOR) pathway inhibitors

Via the activation of the mTOR pathway, HER2 is able to inhibit cell death. Thus, not only the HER2, but also the mTOR pathway presenting good candidates for targeted therapies (Frogne et al. 2009).

The use of mTOR inhibitors are valuable therapeutic tools, namely in combined protocols, in order to reduce resistance development and drugs side effects (T. Liu et al. 2011; Mallon et al. 2011; Tóth et al. 2016). As example, rapamycin is a fungicide macrolide with immunosuppressive and anticancer properties. This compound binds to an immunophilin protein, inhibiting the serine-threonine kinase mTOR and its complex, involved in cell cycle progression and proliferation (Noh et al. 2004; Jhanwar-Uniyal et al. 2019).

1.5.4. Novel compounds for mammary tumour targeted therapies

Current knowledge on HBC describe different tumour subtypes, e.g., the triple-negative, which has no directed therapy (Maniscalco et al. 2013), as well as development of therapeutic resistance, which requires different strategies to improve patients clinical outcome. Highlighting the search for new therapeutic options for cats with mammary tumour, and since few studies exist (Mcdonnel et al. 2013; Samantha et al. 2014), the antiproliferative effects of new compounds, e.g. HDACi (Ediriweera et al. 2019) and MTi (Villanueva et al. 2013), were recently tested in FMC *in vitro* models (CAT-M and FMCp), revealing themselves as promising agents for molecular targeted therapy.

Histone deacetylases are enzymes that control gene expression, and their dysregulation is associated to tumour development (Kamarulzaman et al. 2017; Cui et al. 2018), thus, in the past few years, they have been investigated as potential antitumor agents. In parallel, microtubules are tubulin polymers essentials for cell growth, division and intracellular trafficking (Steinmetz and Prota 2018), and are known to be valuable targets for tumour therapy in human. Interestingly, several HDACis (CI-994, panabinostat, SAHA, SBHA, scriptaid and trichostatin A) and MTis (colchicine, nocodazole, paclitaxel and vinblastine) that have been tested in FMC cell lines showing a dose-dependent antiproliferative effect and conserved molecular and cell death mechanisms, comparing to humans (Yamashita et al. 2003; Almeida et al. 2021).

Moreover, drugs that target epigenetic alterations, such as the DNA methyltransferase inhibitor 5-azacytidine (5-AzaC), were already approved for the treatment of haematological malignancies and are currently being tested in HBC. This kind of treatment was found to be

toxic for cancer cells, but not to healthy mammary cell lines from humans, cats and dogs, validating the therapeutic potential of this drug (Harman et al. 2016), and being one more alternative for the treatment of cats with mammary tumours. Additionally, another important target that has been studied for cancer therapy are the peptidyl arginine deiminase enzymes (PAD), which are involved in citrullination, a translational modification of histones. Recent studies demonstrate that the use of PAD inhibitor, BB-CLA was able to reduce the viability of cancer cells in *in vitro* models of feline and canine mammary tumours, presenting minimal effects on normal cells (Ledet et al. 2018), and promising results for clinical application.

2. Objectives

In the past few years, efforts have been made in the Pathology Lab (FMV – ULisboa) in order to uncover new diagnostic and prognostic biomarkers for cats with mammary carcinoma, and also to discover new therapeutic targets and protocols to improve cats' OS time. Accordingly, the main goal of the research herein presented is to propose new biomarkers and therapeutic protocols for cats with mammary carcinoma. The specific objectives of this project were as follows:

- Map the sequence variants in the feline *her2* gene region encoding for the intracellular tyrosine kinase and the extracellular domains, in order to confirm if any can be used as a biomarker to predict disease progression/prognosis and/or immunotherapy resistance.
- Characterize the antiproliferative effects of tyrosine kinase inhibitors, monoclonal antibodies and antibody-drug conjugate compounds, and mTOR inhibitors by using FMC cell lines (CAT-M, FMCp and FMCm) with different HER2 expression levels.
- Evaluate the leptin and leptin receptor expression in serum and tumour samples from cats with mammary carcinoma, in order to understand its value as a diagnosis/prognosis biomarkers, or therapeutic targets.

It is expected that the results obtained along this research will unveil a deep knowledge about the FMC, as well as define accurate diagnosis biomarkers. Furthermore, it will be tested new therapeutic options, by comparison to the women breast cancer therapeutic protocols, in order to improve cats' prognosis.

Chapter II

Experimental Work

Tyrosine Kinase Inhibitors Are Promising Therapeutic Tools for Cats with HER2-Positive Mammary Carcinoma

Andreia Gameiro, Filipe Almeida, Catarina Nascimento, Jorge Correia and Fernando Ferreira (2021), Pharmaceutics MDPI Journal, DOI: 10.3390/ pharmaceutics13030346 Academic

Abstract

Feline mammary carcinoma (FMC) is a common neoplasia in cat, being HER2-positive the most prevalent subtype. In woman breast cancer, tyrosine kinase inhibitors (TKi) are used as a therapeutic option, by blocking the phosphorylation of the HER2 tyrosine kinase domain. Moreover, clinical trials demonstrated that TKi produce synergistic antiproliferative effects in combination with mTOR inhibitors, overcoming resistance to therapy. Thus, to uncover new chemotherapeutic strategies for cats, the antiproliferative effects of two TKi (lapatinib and neratinib), and their combination with a mTOR inhibitor (rapamycin), were evaluated in FMC cell lines (CAT-M, FMCp and FMCm) and compared with a human breast cancer cell line (SkBR-3). Results revealed that both TKi induced antiproliferative effects in all feline cell lines, by blocking the phosphorylation of EGFR members and its downstream effectors. Furthermore, combined treatments with rapamycin presented synergetic antiproliferative effects. Additionally, the DNA sequence of the *her2* TK domain (exons 18 to 20) was determined in 40 FMC tissue samples, and despite several mutations were found none of them were described as inducing resistance to therapy. Altogether, our results demonstrated that TKi and combined protocols may be useful in the treatment of cats with mammary carcinomas, and that TKi-resistant FMC are rare.

Keywords: Feline mammary carcinoma; HER2; tyrosine kinase inhibitors; targeted therapies; feline *her2* TK mutations

1. Introduction

The feline mammary carcinoma (FMC) is one of the most common feline tumours (12% to 40% of all neoplasms) (Millanta et al. 2005; Maria Soares, Ribeiro, et al. 2016), sharing similar clinicopathological features and histologic subtypes with human breast cancer (Maria Soares, Madeira, et al. 2016), making the cat a suitable model for comparative oncology studies (Vail and Macewen 2000; Porrello et al. 2006; Ranieri et al. 2013; Cannon 2015). Indeed, cats with HER2-positive mammary carcinoma also show a poor prognosis, with high clinical tumour aggressiveness, metastization capability and shorter overall-survival (OS) (Millanta et al. 2005; Vu and Claret 2012; Budiarto 2016; Maria Soares, Ribeiro, et al. 2016), with the overexpression of the epidermal growth factor receptor 2 (HER2) been reported in a range from 33% to 60% of all FMC cases (Soares et al. 2013). Unfortunately, no specific therapeutic targets are available for cats with HER2-positive mammary tumours, being the adjuvant chemotherapy unbeneficial in most of the cases, with the radical surgery being the unique therapeutic strategy (Michishita et al. 2016). Thus, new molecular targets are needed to develop more efficient therapeutic protocols.

In cat, the *her2* gene is localized in the chromosome E1, presenting a sequence identity of 92% with its human counterpart (De Maria et al. 2005; Santos et al. 2013). The HER2 is a tyrosine kinase (TK) transmembrane glycoprotein (186 kDa), constituted by three domains: an extracellular region, a transmembrane domain and an intracellular subdomain with TK activity (Tiwari et al. 2015). In cell membrane, the HER2 can dimerize with itself or with other members of the epidermal growth factor receptor (EGFR) family, showing a proto-oncogene activity, with the ability to modulate cell cycle and apoptotic responses (De Maria et al. 2005; Millanta et al. 2005; Tiwari et al. 2015). Furthermore, HER2 phosphorylation promotes cell proliferation via the RAS-ERK pathway and inhibits cell death via the PI3K-AKT-mTOR pathway, which makes the phosphorylated HER2 a marker of poor prognosis (Frogne et al. 2009) and a useful molecular target in breast cancer therapy. However, 50% of patients with HER2-positive breast cancer show immunotherapy resistance to anti-HER2 monoclonal antibodies, being the use of TK inhibitors (TKi) an alternative approach for these targeted immunotherapies. The TKi are small chemical compounds that prevent HER2 phosphorylation, by competing with the adenosine triphosphate (ATP) at the cytoplasmic catalytic kinase domain and, consequently, blocking the signalling for the downstream cascade activation (Schroeder et al. 2014). Several TKi are conventionally used in human breast cancer therapy, such as lapatinib and neratinib. Accordingly, lapatinib is a reversible dual EGFR inhibitor, binding the ATP-binding pocket of HER1 and HER2 proteins (Shi et al. 2016), presenting apoptotic effects (Schroeder et al. 2014). This compound has a specific binding location, encoded by exon 20 of the *her2* gene, with several point mutations being associated with therapy resistance (e.g., L755S, T798M)

(Sun et al. 2015). In parallel, neratinib is an irreversible pan-HER inhibitor that interacts with HER1, HER2 and HER4 proteins (Tiwari et al. 2015), able to overcome lapatinib therapy resistance (Sun et al. 2015). This molecule binds the TK ATP binding pocket, with a high binding specificity in a conserved cysteine (Cys) residue (Kong and Feldinger 2015), leading to cell death, by a ferroptosis mechanism (Lu et al. 2018; Nagpal et al. 2019), with woman breast cancer patients having the acquired mutation at the residue T798I in HER2 and/or HER4 overexpression showing neratinib resistance (Hanker et al. 2017). Indeed, the HER4 protein is crucial for normal mammalian gland development and differentiation, and it is hypothesized that HER2-positive tumour cells can shift their dependency from HER2 to HER4 (Canfiel et al. 2015), to maintain cell survival and growth.

Nowadays, the combined treatments, such as the use of TKi conjugated with the mammalian target of rapamycin (mTOR) inhibitor, are valuable therapeutic tools, in order to reduce resistance development and drug secondary effects. Indeed, the rapamycin that is a fungicide macrolide with immunosuppressive and anticancer properties, binds to an immunophilin protein family, inhibiting the serine-threonine kinase mTOR (Noh et al. 2004; Jhanwar-Uniyal et al. 2019) and its complex (mTORC) involved in cell cycle progression and cell proliferation (Jhanwar-Uniyal et al. 2019), two hallmarks of the carcinogenesis process (Noh et al. 2004).

For breast cancer patients, treatments based on precision and target specificity are crucial towards the improvement of the therapeutic response and preventing drug resistance (Shi et al. 2016). Once no data is available about the use of these compounds in feline mammary carcinoma, this study aims to: 1) evaluate the antiproliferative effects of two TKi (lapatinib and neratinib) using three feline mammary carcinoma cell lines (CAT-M, FMCp and FMCm); 2) characterize the effect of the TKi in the phosphorylation patterns on EGFR family members (HER1, HER2 and HER4), its downstream pathways (PI3K/AKT and ERK1/2) and on the tumour suppressor PTEN protein; 3) measure the cytotoxic effects of the mTOR inhibitor, rapamycin and evaluate its effect on the mTOR phosphorylation pattern; 4) evaluate the synergistic antiproliferative effects of the combined treatments of using TKi and rapamycin; and 5) identify genomic mutations in the feline *her2* gene TK domain in FMC tissue samples, in order to assess their use as prognostic/drug-resistance biomarkers.

2. Materials and Methods

2.1. Feline and human mammary carcinoma cell lines

In this study, three feline mammary carcinoma cell lines (CAT-M, FMCp and FMCm) were immunophenotyped and tested, as well as a HER2-overexpressing human breast cancer cell line (SkBR-3) used as positive control (**Table S1.1 – Annex I**). CAT-M and SkBR-3 cell lines were maintained in Dulbecco's modified Eagle Medium (DMEM; Corning, New York, NY,

USA), whereas FMCp and FMCm cell lines were growing in Roswell Park Memorial Institute 1640 Medium (RPMI; Corning), both supplemented with heat-inactivated 20% (*v*/*v*) fetal bovine serum (FBS; Corning), and incubated at 37°C, in a humidified atmosphere of 5% (*v*/*v*) CO_2 (Nuaire, Plymouth, MN, USA). Periodically, cell lines were tested for mycoplasma (MycoSEQ™ Mycoplasma Detection Kit, Thermo Fischer, Waltham, MA, USA) and examined for their morphology and proliferation rate.

2.2. In vitro cytotoxicity assays

To determine the effect of the TKi (lapatinib and neratinib; Sigma-Aldrich, Darmstadt, Germany) and of the mTOR inhibitor (rapamycin; Sigma-Aldrich), viability assays were performed using the Cell Proliferation Reagent WST-1 kit (Abcam, Cambridge, UK), following to the manufacturer's instructions. Briefly, cell lines were seeded in 96-well plates to reach a 90% confluency at 24 hours. Then, cells were exposed to increasing concentrations of each drug to obtain a dose-response plot (**Table 2**), while control wells were left unexposed. Dymetilsulfoxide (DMSO; Sigma-Aldrich) was used as vehicle control, at a maximum final concentration of 1%. After 72 hours of drug exposure, the WST-1 reagent (Abcam) was added during an incubation period of 4 hours, at 37°C, and absorbance at 440 nm was measured using a plate reader (FLUOStar Optima, BMG LabTech, GmbH, Ortenberg, Germany). Triplicate wells were used to determine each data point and three independent experiments were performed.

In combined treatments (lapatinib plus rapamycin; neratinib plus rapamycin) the same strategy was followed, with the concentrations chosen in a range that a small or no cytotoxic effect was observed.

Table 2. Concentrations of lapatinib (nM), neratinib (nM) and rapamycin (nM), used in the cytotoxicity assays. Cells were exposed to drugs for 72 hours before cytotoxicity evaluation.

Drug concentrations for the cytotoxicity assays		
Lapatinib (nM)	**Neratinib (nM)**	**Rapamycin (nM)**
195	0.195	0.195
390	0.39	0.39
780	0.78	0.78
1560	1.56	1.56
3125	3.125	3.125
6250	6.25	6.25
12,500	12.5	12.5
25,000	25	25
50,000	50	50
100×10^3	100	100
250×10^3	250	250

500×10^3	500	500
1×10^6	750	750
	1000	1000
	1500	1500

2.3. Immunoblotting

For the quantification of protein expression levels, the western blot technique was performed as previously reported by us (Maria Soares, Ribeiro, et al. 2016; Almeida et al. 2021), and other groups (Chiang and Abraham 2005; Okita et al. 2015). Briefly, for the preparation of whole cell extracts, cell lines were seeded in 6-well plates, in order to obtain a confluency of 90%, after 24 hours. Then, cells were exposed to drugs, using IC_{50} concentrations, and a negative control was left unexposed, using DMSO (Sigma-Aldrich) as a vehicle. After 72 hours of exposure, cells were collected using 200 µL of RIPA lysis buffer (50 mM TrisHCl, 150 mM NaCl, 1% Tween-20, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, pH 8.0) supplemented with 1% 100X Halt™ protease inhibitor cocktail EDTA-free (Thermo Fischer) and 2 mg/mL iodoacetamide (AppliChem, GmbH, Darmstadt, Germany). Afterwards, whole cell extracts were resuspended with 10 µL of SDS-PAGE Loading Buffer 5X (NZYTech, Lisbon, Portugal), denatured for 10 minutes at 96°C, and stored at −80°C, until further use. Before immunoblotting, the extracts were fully thawed, and then resolved in 7.5% SDS-PAGE. Proteins were transferred into nitrocellulose membranes (Amersham Protran 0.45 NC, GE Healthcare, Chicago, Il, USA) and blocked in 5% (*w*/*v*) dried skimmed milk diluted in phosphate buffered saline (PBS)−0.1% (*v*/*v*) Tween-20 (National Diagnostics, Atlanta, GA, USA). Membranes were probed with primary antibodies (Abcam; **Table 3**) diluted in 1% (*w*/*v*) dried skimmed milk in PBS−0.1% Tween-20 (National Diagnostics), overnight at 4°C, and then with horseradish peroxidase (HRP)-conjugated secondary antibodies (Goat HRP anti-rabbit IgG H&L and goat HRP anti-mouse IgG H&L, 1:10,000, Abcam) for 1 hour at room temperature (RT). Bands were detected using ECL (Clarity Substrate, Bio-Rad, Hercules, CA, USA), visualized and analysed using a Chemidoc XRS+ system with Image Lab capture software (Bio-Rad).

Table 3. Primary antibodies and their dilutions used for chemoluminescence analysis.

Primary antibody	Clone	Dilution
β-actin	AC-15	1:5000
HER2	CB11	1:1000
HER2 pY1221 + Y1222	Polyclonal	1:1000
HER1 pY1173	E124	1:500
HER4 pY1284	Polyclonal	1:500
AKT1 pS473	EP2109Y	1:2000

ERK1/2 pT202/pY204 + pT185/pY187	MAPK-YT	1:5000
PTEN pT366	EP229	1:1000
mTOR pS2448	EPR426(2)	1:10,000

2.4. Animal population

The forty cats diagnosed with mammary carcinoma enrolled in this study, were recruited at the Hospital of Faculty of Veterinary Medicine, University of Lisbon, being the surgical procedures consented by the owners, and without interfering with the animals' well-being. Their clinical history was fully documented (**Table 4**) (Maria Soares, Ribeiro, et al. 2016), including breed, age, reproductive status and contraceptive administration, treatment (surgery, or surgery plus chemotherapy), number, location and size of tumour lesions, histopathological classification, tumour immunophenotype (Maria Soares, Madeira, et al. 2016; M. Soares, Ribeiro, Carvalho, et al. 2016), malignancy grade (Elston, C.W. and Ellis 1998), presence of tumour necrosis, lymphatic invasion, lymphocytic infiltration, cutaneous ulceration, regional lymph node involvement and clinical stage (TNM system). All tissue samples were frozen at −80°C and stored until further use.

Table 4. Clinicopathological features of female cats with mammary carcinomas enrolled in this study (n= 40).

Clinicopathological feature	Number (%)	Clinicopathological feature	Number (%)
Breed		**Age**	
Indeterminate	33 (82.5%)	<8years old	3 (7.5%)
Siamese	4 (10%)	≥8 years old	37 (92.5%)
Persian	2 (5%)	**Tumour size**	
Norwegian Forest	1 (2.5%)	<2cm	9 (22.5%)
Spayed; 1 unknown		2–3cm	19 (47.5%)
Yes	19 (47.5%)	>3cm	12 (30%)
No	20 (50%)	**HP* classification**	
Contraceptives; 7 unknown		Tubulopapillary carcinoma	8 (20%)
Yes	23 (57.5%)	Solid carcinoma	9 (22.5%)
No	10 (25%)	Cribiform carcinoma	5 (12.5%)
Treatment		Mucinous carcinoma	5 (12.5%)
Mastectomy	36 (90%)	Tubular Carcinoma	11 (27.5%)
Mastectomy+Chemo	4 (10%)	Papillary-cystic carcinoma	2 (5%)
Multiple tumours		**HP* Malignancy grade**	
Yes	31 (77.5%)	I	2 (5%)
No	9 (22.5%)	II	5 (12.5%)
Regional lymph node status; 2 unknown		III	33 (82.5%)
Positive	14 (35%)	**Tumour necrosis**	
Negative	24 (60%)	Yes	29 (72.5%)
Stage (TNM classification)		No	11 (27.5%)
I	9 (22.5%)	**Lymphatic invasion**	

II	7 (17.5%)	Yes	5 (12.5%)
III	21 (52.5%)	No	35 (87.5%)
IV	3 (7.5%)	**Lymphocytic infiltration**	
Mammary location		Yes	27 (67.5%)
M1	11 (27.5%)	No	13 (32.5%)
M2	8 (20%)	**Tumour ulceration**	
M3	14 (35%)	Yes	3 (7.5%)
M4	11 (27.5%)	No	37 (92.5%)
fHER2 status		**Ki-67 index**	
Positive	12 (30%)	Low (<14%)	30 (75%)
Negative	28 (70%)	High (≥14%)	10 (25%)
ER status		**PR status**	
Positive	12 (30%)	Positive	20 (50%)
Negative	28 (70%)	Negative	20 (50%)

*HP – Histopathological; TNM – Tumour, Node, Metastasis; ER – Oestrogen receptor; PR – Progesterone receptor

2.5. DNA extraction, amplification and sequence analysis of the feline her2 TK domain

Genomic DNA extraction was performed in the three feline cell lines, collected after reaching confluence in a T25 culture flask, and in 5 mg of 44 frozen tissue samples (40 feline mammary tumour samples and 4 breed controls), as previously described (Santos et al. 2009; Ferreira, Soares, et al. 2019; Nascimento et al. 2020), using a QIAmp FFPE kit (Qiagen, Dusseldorf, Germany) and following the manufacturer's recommendations. Tissue samples were homogenized with the Tissue Lyser II (Qiagen), and all samples were digested with protease K (20 mg/mL; Qiagen). After several washing steps, the genomic DNA was eluted from the extraction columns and its quality and quantity was measured in NanoDrop ND-100 Spectrophotometer (Thermo Fischer Scientific). The feline *her2* TK domain (NC_018736.3) was identified by comparison with the genomic human *her2* sequence (NC_000017.11), and primers for DNA amplification of exons 18 to 22 were designed in the Primer designing tool (NCBI, Bethesda, MD, USA; **Table 5**). PCR technique was performed with a standard reaction mixture (4 µL/sample of Phusion GC buffer from Thermo Fischer Scientific; 0.4 µL/sample of dNTPs from Grisp, Porto, Portugal; 0.1 µL/sample of each forward and reverse primer and 0.2 µL/sample of DNA polymerase from Thermo Fischer Scientific) at a final DNA concentration of 4 ng/mL. For amplification of the exons 18 and 19 of the *her2* gene, PCR reactions were performed in a PCR Thermal Cycler (VWR Thermocycler, Leicestershire, England) as follows: denaturation at 98°C for 30 seconds, followed by 35 cycles at 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 10 seconds, plus one final extension step at 72°C for 10 minutes. For exons 20 to 22, the melting temperature was 58°C. After confirmation of the expected size for each amplified sequence in a 2% agarose gel (Sigma-Aldrich), DNA fragments were purified and sequenced by Sanger technique (StabVida, Almada, Portugal). DNA sequences were inspected for inaccuracies.

Table 5. Primers for genomic DNA amplification and sequencing, covering the feline *her2* TK domain, between exon 18 to exon 22.

Exons	Forward (5′-3′)	Reverse (5′-3´)
18 and 19	CTAGTGGAGCCATGCCCAA	GGAGGTCCCTCCTGTACTCC
20	AATCTTGGACGTAAGCCCCTC	AGGCCCCCTAAGTGCATACC
21 and 22	CTGACATCCACCGTGCAGTT	CGTAGCTCCACACGTCACTC

The feline sequenced samples were aligned with the identified feline *her2* (NC_018736.3), using the ClustalW tool (BioEdit Alignment Editor software) and the consensus sequence was confirmed using SeqTrace 9.1 software (Stucky 2012), while single nucleotide polymorphisms (SNP) and protein amino acid changes were identified by using Expert Protein Analysis System (ExPASY) translate tool and compared with the original protein sequence (NP_001041628.1, NCBI). Mutations identified in the feline tissue samples were compared to the human *her2* sequence (NC_000017.11) and searched in National Cancer Institute, International Cancer Genome Consortium and Catalogue of Somatic Mutations in Cancer (COSMIC) databases for putatively induced resistance to the TKi tested in this study.

2.6. Statistical analysis

Statistical analysis was carried out using the GraphPad Prism software (version 5.04, for Windows, San Diego, CA, USA), with two-tailed *p*-values less than 0.05 being considered statistically significant, and a 95% confidence interval (*p< 0.05, **p< 0.01 and ***p< 0.001). Regarding the cytotoxicity assays, outliers with more than two standard deviations were removed and the IC_{50} value for each drug was calculated using Log (Inhibitor) *vs.* Response (Variable slope) function. For the drug conjugation assays, the two-way ANOVA test was performed. In the animal population, correlations considering the genomic mutations were assessed between groups using the non-parametric Mann Whitney test.

3. Results

3.1. Lapatinib and neratinib exert antiproliferative effects in all feline mammary carcinoma cell lines

Results showed that incubation of FMC cell lines with lapatinib or neratinib, exert potent antiproliferative effects in a dose-dependent fashion. Indeed, the lapatinib was able to induce 100% of cytotoxicity in all feline cell lines (CAT-M with a IC_{50}= 3930 nM±49, **Figure 6A**; FMCp with a IC_{50}= 4870 nM±100, **Figure 6B**; FMCm with a IC_{50}= 17,470 nM±100, **Figure 6C**), while neratinib showed lower cytotoxicity (33.5% of cytotoxicity in CAT-M cells, **Figure 6A**; 79.4% of cytotoxicity in FMCp cells, **Figure 6B**; and 31.4% of cytotoxicity in FMCm, **Figure 6C**). As expected, the human breast cancer SkBR-3 cell line showed 91.1% of cytotoxicity when

incubated with lapatinib (IC_{50}= 16,220 nM±1040) and a cytotoxicity of 60.5% in the presence of neratinib (**Figure 6D**).

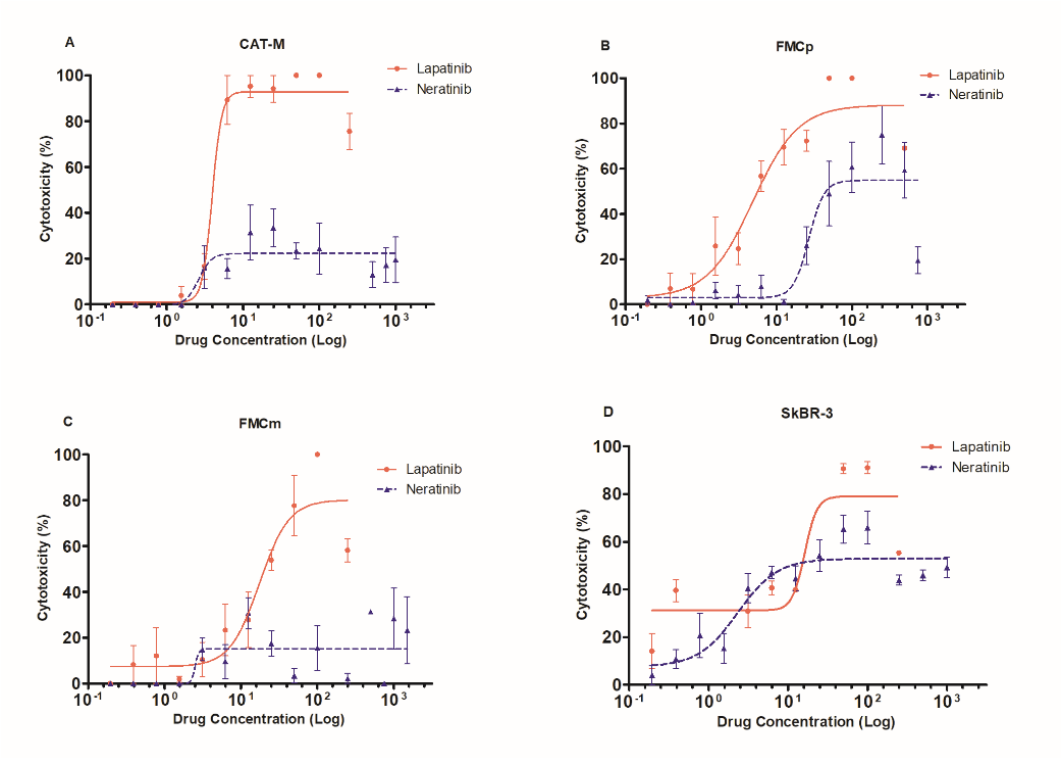

Figure 6. Lapatinib and neratinib showed strong cytotoxic effects in feline mammary carcinoma cell lines. **A)** CAT-M cell line presented higher cytotoxic effects when incubated with lapatinib (IC_{50}= 3930 nM±49) than with neratinib. **B)** FMCp cell line was susceptible to both lapatinib and neratinib (100% and 74.9% of cytotoxicity, respectively; IC_{50}= 4870 nM±100 for lapatinib). **C)** FMCm cell line also showed high cytotoxic effects in the presence of lapatinib (IC_{50}= 17,470 nM±100), contrasting with the neratinib. **D)** SkBR-3 cells showed a maximum cytotoxic effect of 91.1% (IC_{50}= 16,220 nM±1040) after exposure to lapatinib and 66.0% when exposed to neratinib. For graphical convenience, lapatinib was represented in a µM range, while neratinib was defined in a nM range. The experiments were performed in triplicates, in three independent assays.

3.2. Lapatinib and neratinib inhibit the phosphorylation of HER2 and its downstream cascade

Considering the promising results obtained from the incubation of all feline cell lines to both TKi, the HER2 expression was evaluated by immunoblot analysis. Results revealing that HER2 expression is much lower in feline cell lines than in human positive control (SkBR-3), being the cell line FMCp HER2-negative (**Figure 7A**). Once the HER2 expression in feline cell lines was higher in CAT-M cells, further analysis was performed to characterize the effects of TKi on the HER2 signalling pathway activity using this cell line. After lapatinib exposure, a decreasing on the phosphorylation levels of HER1 and HER2 was detected, coupled with a reduction in the phosphorylation levels of the downstream effectors AKT and ERK1/2. Moreover, an increase in phosphorylated PTEN at Thr366 was also observed (**Figure 7B**). Corroborating the lower cytotoxicity induced by neratinib incubations, differences in the phosphorylated levels were less noticeable (**Figure 7B**).

Interestingly, when feline tumour cells were incubated with TKi, in particularly, with lapatinib, the HER2 expression levels increased, both in CAT-M (**Figure 7C**) and FMCp (**Figure 7D**) cell lines.

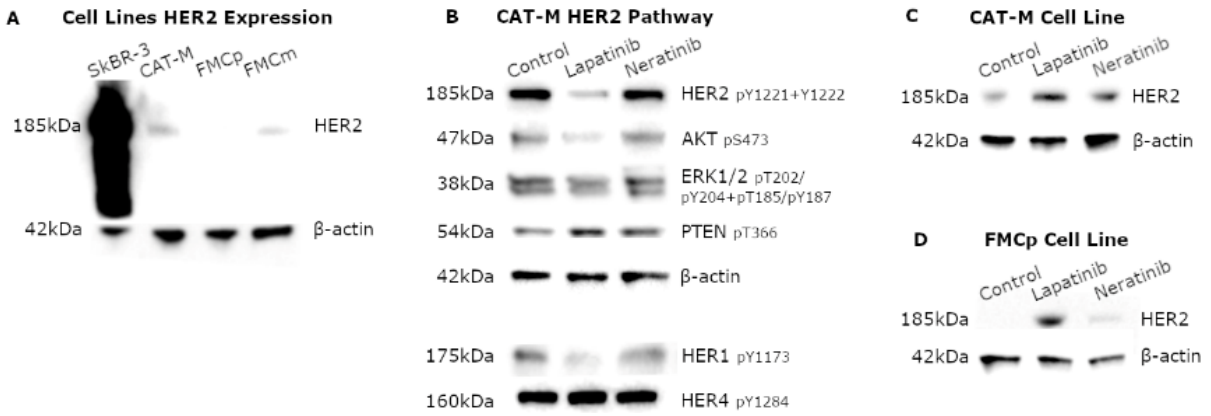

Figure 7. Feline cell lines presented low HER2 expression, showing altered phosphorylation levels of HER2 and its downstream effectors after TKi exposure. **A)** CAT-M and FMCm cell lines presented low HER2 expression levels, with no expression detected in the FMCp cell line. **B)** After exposure to lapatinib, the CAT-M cell line showed decreased phosphorylation levels of the HER1 (pY1173), HER2 (pY1221+Y1222) and of its downstream effectors, AKT1 (pS473) and ERK1/2 (pT202/pY204+pT185/pY187), coupled with an increase of PTEN phosphorylation at Thr366. Results were less perceptible when cells were exposed to neratinib. **C)** TKi exposure, particularly, lapatinib, increased HER2 levels not only in CAT-M cells, but also in **D)** FMCp cells. β-actin was used as loading control.

3.3. Rapamycin does not induce strong antiproliferative effects in feline cell lines

The use of rapamycin as a single drug did not presented a valuable antiproliferative effect (**Figure 8A**). Accordingly, the CAT-M and FMCm cell lines showed 38.7% and 41.3% of cytotoxicity, respectively, with the FMCp showing slightly more sensitivity to rapamycin (43.3% of cytotoxicity). Similar results were obtained with the control SkBR-3 cell line (26.9% of cytotoxicity). Additionally, the immunoblot analysis revealed a decrease in the mTOR phosphorylation levels after rapamycin exposure (**Figure 8B**).

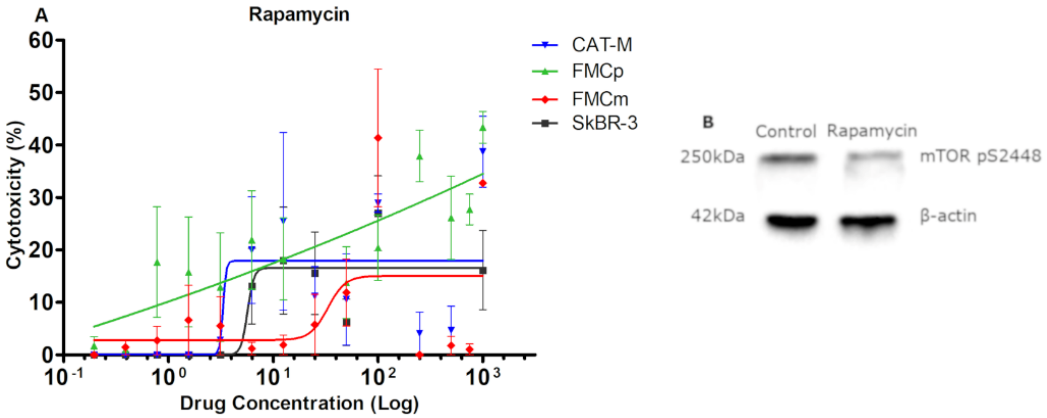

Figure 8. Rapamycin showed small cytotoxicity in all tested tumour cell lines, although it lead to a decrease in the phosphorylation levels of mTOR at S2448. **A)** The most susceptible cell line to rapamycin exposure was FMCp (43.4% of cytotoxicity) followed by the FMCm (41.3% of cytotoxicity) and the CAT-M cell lines (38.7% of cytotoxicity). The human SkBR-3 cell line was used as control,

presenting a maximum of 29.6% of cytotoxicity. The experiments were performed in triplicates and repeated three times. **B)** Rapamycin reduces the phosphorylation levels of the mTOR at S2448. β-actin was used as loading control.

3.4. Combined exposures of TKi and mTOR inhibitor showed strong synergistic antiproliferative effects

The combined exposures of lapatinib plus rapamycin and neratinib plus rapamycin, demonstrated high synergistic cytotoxic effects regardless of feline cell lines. For instance, in the CAT-M cell line, the cytotoxic effects increased from 5.6% to 57.5% (p= 0.0360) by adding 6.25 nM of rapamycin to 3125 nM of lapatinib and raised from 2.5% to 49.9% (p= 0.0044) when cells were incubated with 12.5 nM of neratinib plus 6.25 nM of rapamycin (**Figure 9A**). Similarly, in FMCp cell line, the antiproliferative effects increased from 6.9% to 54.4% (p< 0.001) when lapatinib at 780 nM was combined with rapamycin at 6.25 nM and from 0.4% to 44.5% (p= 0.0034) when neratinib at 3.125 nM was combined with rapamycin at 6.25 nM (**Figure 9B**). In the metastatic FMCm cell line, the higher antiproliferative effects were obtained by the use of lapatinib at 22,650 nM plus rapamycin at 6.25 nM, increasing from 9.6% to 95.2% (p< 0.001), and exposing cells to neratinib at 25 nM plus rapamycin at 6.25 nM, increasing the antiproliferative effects from 9.6% to 76.3% (p< 0.001; **Figure 9C**). In the control SkBR-3 cell line, also synergistic effects were detected in all combination assays (**Figure 9D**).

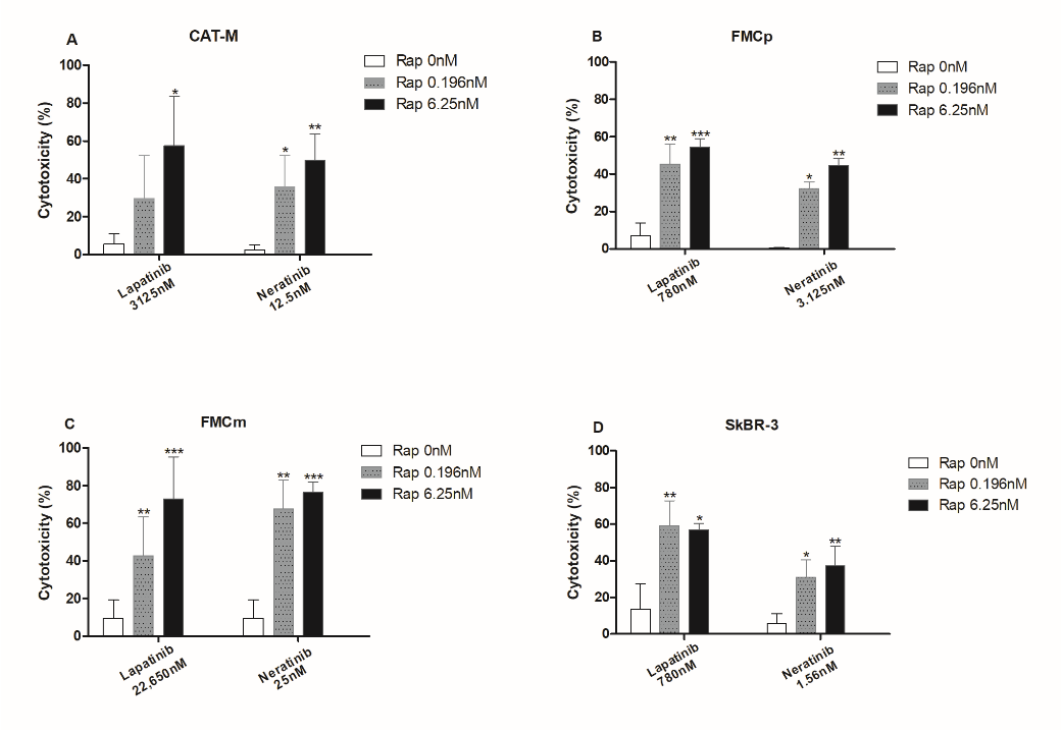

Figure 9. Combinations of TKi and mTOR inhibitor (lapatinib plus rapamycin and neratinib plus rapamycin) showed synergistic antiproliferative effects in feline mammary tumour cell lines. **A)** CAT-M cells presented a significantly higher antiproliferative response when exposed to lapatinib at 3125 nM plus rapamycin at 6.25 nM or incubated with neratinib at 12.5 nM plus rapamycin at 6.25 nM (57.5% of cytotoxicity, *p< 0.05; 49.9% of cytotoxicity, **p< 0.01, respectively). **B)** FMCp cells showed significant synergistic effects of conjugations between lapatinib at 780 nM plus rapamycin at 0.196 nM or at 6.25

nM (45.5% of cytotoxicity, ** $p < 0.01$; 54.4% of cytotoxicity, *** $p < 0.001$, respectively), and when neratinib at 3.125 nM plus rapamycin at 0.196 nM or at 6.25 nM were used (32.1% of cytotoxicity, * $p < 0.05$; 44.5% of cytotoxicity, ** $p < 0.01$, respectively). **C)** FMCm cells presented an increased cytotoxic response to the conjugations of lapatinib at 22,650 nM plus rapamycin at 0.196 nM or at 6.25 nM (42.6% of cytotoxicity, ** $p < 0.01$; 95.2% of cytotoxicity, *** $p < 0.001$, respectively) and neratinib at 25 nM plus rapamycin at 0.196 nM or at 6.25 nM (67.7% of cytotoxicity, ** $p < 0.01$; 76.3% of cytotoxicity, *** $p < 0.001$, respectively). **D)** SkBR-3 cells used as control, displayed similar antiproliferative responses to feline cell lines. All the assays were performed in triplicate and repeated a total of three separated times.

3.5. Mutations found in the her2 TK domain of FMC clinical samples were not associated with TKi therapy resistance

Previous studies on somatic mutations in the TK domain of the *her2* gene, in human breast cancer patients, revealed several mutations associated with therapy resistance to TKi and/or specific clinicopathological features, reported in National Cancer Institute, International Cancer Genome Consortium and COSMIC databases. In this work, the mutational analysis of the feline *her2* TK domain, showed that mutations occur in the majority of tumour samples (90%, 36/40), being identified a total of 42 single variants (SVs; **Figure 10**), located in introns (54.8%; 23/42) and exons (45.2%; 19/42; **Table S1.2 – Annex I**). Regarding the intronic SVs, 21.7% (5/23) were found in splicing regions (c.19631 and c.19643; c.20278 and c.20289; c.20612), with the majority detected between exons 18 and 19 (60.9%; 14/23). Further sequence analysis revealed that exons 18 and 20 showed a higher number of mutations (57.9%, 11/19 and 36.8%, 7/19, respectively), contrasting with the exon 22 that has only one mutation with a very high frequency (85%; 34/40; c.20940 T>G) and with exons 19 and 21 where no mutations were detected. Finally, when tumour samples were divided into four molecular subtypes, 71.4% of the triple-negative tumours (5/7), 58.3% of the HER2-positive (7/12) and 55.6% of the luminal B (10/18) showed at least one mutation. No mutations were detected in luminal A tumours (0/3).

A special consideration was made at exon 20, which encodes for the local binding of the TKi. In this exon 7 mutations were reported, in a total of 13 samples, and occurring 46.2% (6/13) in the HER2-positive tumour subtype. Furthermore, evaluating the SVs, 4 missense mutations (c.20380 C>G; c.20384 A>T; c.20428 G>C; c.20459 A>T), 2 synonymous mutations (c.20382 T>C; 20436 G>A) and 1 base change that leads to a STOP codon (c.20385 T>G) were observed.

After comparing the feline mutations with the genomic human *her2* sequence, and searching in the referred databases, none of the mutations were reported as inducing resistance to TKi tested.

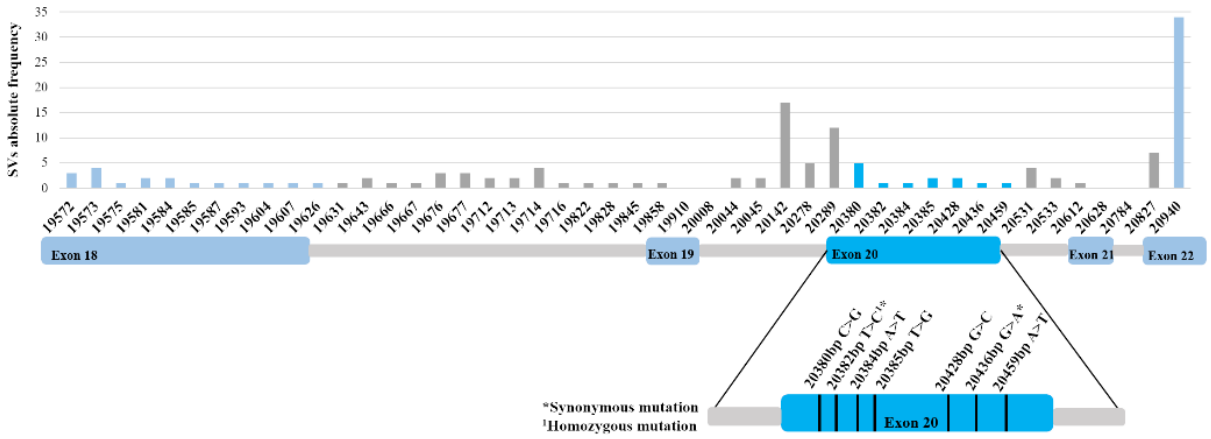

Figure 10. Mutations identified in feline *her2* TK domain are not reported to induce therapeutic resistance in woman breast cancer. Most of the detected mutations were localized at intronic regions, although some of them were identified in exons, as a very frequent mutation in the exon 22 (c.20940 T>G; 85%; 34/40). Regarding the exon 20 that encodes for the protein region recognized by TKi, none of the seven identified mutations were reported as being related to therapeutic resistance in woman breast cancer.

In order to identify new prognostic biomarkers, correlations were made between the SVs reported and animals' clinicopathological features. This analysis did not reveal any correlation, beyond the mutation c.19573 A>T, located in exon 18 associated with tumour size (*p= 0.045; **Figure 11**), revealing that the mean rank of the general samples was 2.64 cm±1.13, comparing with 4.13 cm±1.44, of the samples presenting that mutation.

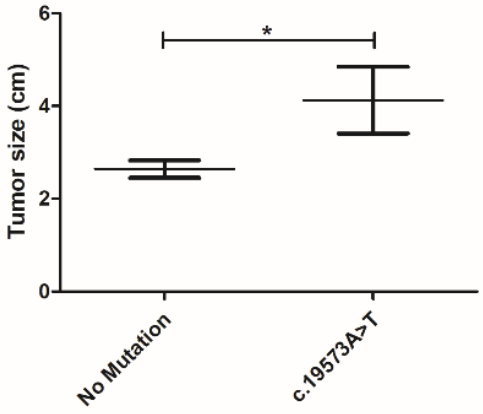

Figure 11. A significant positive correlation was found between the tumour size and the presence of c.19573 A>T mutation in exon 18 (*p= 0.045). The mutation c.19573 A>T was associated to larger tumour sizes (4.13 cm±1.44 *vs.* 2.64 cm±1.13).

4. Discussion

Feline mammary carcinoma is usually diagnosed belatedly, presenting an aggressive behaviour, with the FMC HER2-positive showing a poor prognosis (Vu and Claret 2012; Maria Soares, Madeira, et al. 2016; M. Soares, Correia, Peleteiro, et al. 2016). Therefore, an early detection and an effective therapy is crucial, in order to improve survival time in cats with mammary tumours. Thus, in this study, the antiproliferative effects of two TKi (lapatinib and

neratinib) were evaluated using three FMC cell lines (CAT-M, FMCp and FMCm), with different HER2 expression levels and no mutations described as possibly conducing to resistance to therapy (**Table S1.3 – Annex I**). Moreover, the obtained results were compared to a human HER2-overexpressing breast cancer cell line (SkBR-3).

In therapeutic protocols, TKi inhibit the phosphorylation of EGFR family members, blocking its downstream pathways, such as lapatinib. This TKi was approved for solid, and metastatic HER2-positive breast tumours (Frenel et al. 2009; Opdam et al. 2012) preventing the phosphorylation of HER1 (T1173) and HER2 (T1221/1222), markers of poor prognosis in breast cancer patients (Frogne et al. 2009; Canfiel et al. 2015). Lapatinib was reported as inducing an antiproliferative effect in cells expressing different HER2 concentrations (Segovia-Mendoza et al. 2015), as proved in this assay, being the HER2-positive CAT-M cells the most susceptible (IC_{50}= 3930 nM±49), with similar results obtained in the SkBR-3 cell line (IC_{50}= 16,220 nM±1040) (Hegde et al. 2007; Scaltriti et al. 2009; Formisano et al. 2014; Matsumoto et al. 2018). Although, 100% of cytotoxicity was also observed in the HER2-negative FMCp cell line (IC_{50}= 4870 nM±100). Indeed, studies in humans demonstrated that lapatinib is useful in patients with triple-negative breast tumours, by activating NF-kB and the anti-apoptotic Bcl-2 protein (Chen et al. 2013), sensitizing tumour cells to the annexin A6 upregulation (Widatalla et al. 2019) and inducing apoptosis (Liu et al. 2016). Furthermore, lapatinib also interacts with HER1, which is usually upregulated in triple-negative tumours, being suggested as an alternative therapeutic tool for patients with this breast cancer subtype (Nielsen et al. 2004; Bouchalova et al. 2009). In parallel, the metastatic cell line (FMCm) showed the highest IC_{50} value (17,470 nM±100), suggesting that in metastatic tumours, lapatinib is more useful when combined with other compounds (Frenel et al. 2009; Opdam et al. 2012). In addition, the molecular effects of lapatinib seem to be conserved between human and feline cell lines, with CAT-M cells showing lower phosphorylation levels of HER2 (Y1221+Y1222), HER1 (Y1173), AKT (S473) (Manning and Toker 2017; Matsumoto et al. 2018) and ERK1/2 (T202/Y204+T185/Y187) (Xia et al. 2002; Tanizaki et al. 2011; Formisano et al. 2014; Appert-Collin et al. 2015; Ma et al. 2017) after lapatinib exposure. Results obtained also demonstrated that lapatinib increased the phosphorylation levels of PTEN at T366, putatively through the glycogen synthase kinase 3 (GSK-3), playing a role in the destabilization of the protein, which leads to a more potent effect in the suppression of cancer cells proliferation (Maccario et al. 2007). Additionally, as previously reported in humans, lapatinib also increased the HER2 expression levels, possibly by stabilizing the protein at the cell membrane and inhibiting its ubiquitination (Scaltriti et al. 2009). Interestingly, this effect was also observed in the HER2-negative FMCp cell line, which could support the antiproliferative effects obtained by lapatinib.

The other TKi tested in this study was neratinib, a compound approved for the adjuvant treatment of early-stage and metastatic HER2-positive breast cancer (Food and Drug

Administration). Interestingly, this TKi interacts with different EGFR family members (HER1, HER2 and HER4), inducing ubiquitination and lysosomal degradation of the HER2 (Y. Zhang et al. 2016), and it is able to reverse membrane-bound ATP transporters surpassing multidrug resistance (Zhao et al. 2012). In the neratinib assay cytotoxic effects were reported for all the FMC cell lines. Indeed, CAT-M cell line presented a maximum of 33.5% of cytotoxicity, smaller when compared to the human, SkBR-3 cell line (60.5% of cytotoxicity) (Y. Zhang et al. 2016), possibly due to CAT-M cells' lower HER2 expression levels. Notably, the FMCp cell line presented a high sensitivity to neratinib (74.9% of cytotoxicity), suggesting its effect in other EGFR family members (McGowan et al. 2013; Nagpal et al. 2019), and supporting the use of neratinib in HER2-negative breast cancer (Mullooly et al. 2015). On the other hand, the metastatic FMCm cell line, revealed to be the less susceptible to neratinib (31.4% of cytotoxicity), suggesting its use as adjuvant in metastatic tumours (Crown et al. 2012; Collins et al. 2019). Furthermore, in this cell line, the use of neratinib did not demonstrate a dose-dependent effect. This kind of behaviour is documented for humans, e.g., by NmU overexpression (Rani et al. 2014), a protein involved in breast cancer progression and metastization (Garczyk et al. 2017), or by an increased activity of the enzyme cytochrome P4503A4 (Breslin et al. 2017), leading to resistance to therapy. Additionally, after exposing cells to neratinib, it was not possible to demonstrate its molecular effects. In fact, only a slightly decrease in the phosphorylation levels of HER1 (Y1173) (Rabindran et al. 2004) were observed. Moreover, an increase in HER2 was obtained by exposure to neratinib, suggesting its stabilization at the cell membrane, by preventing protein phosphorylation.

Another valuable target in tumour therapy is the mTOR pathway, described as being hyperactivated in 80% of human cancers (Watanabe et al. 2011), and particularly in cats mTOR is involved in metastization, invasion and tumour progression (Maniscalco et al. 2013). The use of a mTOR inhibitor, rapamycin, in FMC cell lines revealed weak antiproliferative effects, similarly to the human SkBR-3 cell line, and as reported in humans (Noh et al. 2004; Watanabe et al. 2011; Zheng et al. 2012). However, the highest cytotoxic effect was obtained for the FMCp cell line (43.3% of cytotoxicity), corroborating the data that mTOR is frequently overexpressed in HER2-negative breast cancer (Walsh et al. 2012). Moreover, our results showed that the cytotoxicity of rapamycin did not demonstrate a dose-dependent effect, suggesting that cells were able to do the efflux of rapamycin, as reported in humans, by the overexpression of the ABCB1 transporter (Wang et al. 2020). Additionally, evaluating the mTOR (S2448), using the CAT-M cell line as example, a decrease in its phosphorylation pattern was observed after exposure to rapamycin, as documented for women (Noh et al. 2004; B. Liu et al. 2011; Zhang et al. 2018). Since in breast cancer therapy several advantages are known to result from the combined treatments (T. Liu et al. 2011; Kong and Feldinger 2015), and after the characterization of all the above compounds a synergistic effect of its

combinations were demonstrated in the feline mammary carcinoma cell lines. The major advantage of this combined protocols is to block simultaneously different cell pathways, and overcome acquired resistant patterns, as documented for lapatinib, e.g., by HER3 (Noh et al. 2004) or HER4 activated pathways (Canfiel et al. 2015), cell signal reprogramming (Ruprecht et al. 2017), recovering of the AKT/mTOR pathway (L. Liu et al. 2009), or Src-dependent resistance (Formisano et al. 2014). Furthermore, also for the TKi neratinib, acquired resistance is described, e.g., by NmU overexpression (Rani et al. 2014), or the reactivation of the proto-oncogene YES-1, a member of the Src family (Takeda et al. 2020). In the combined protocols, the best results were achieved in the FMCm cell line, with the highest increase in the cytotoxic effect occurring by the combination of lapatinib at 22,650 nM plus rapamycin at 6.25 nM, with an increase of 85.6% of cytotoxicity (from 9.6% to 95.2%), and for the combination of neratinib at 25 nM plus rapamycin at 6.25 nM, where an increase of 66.7% of cytotoxicity (from 9.6% to 76.3%) was obtained. The reported results, suggest the use of combined protocols as a valuable tool for feline mammary tumour therapy, as described for women breast cancer patients (T. Liu et al. 2011; Kong and Feldinger 2015), particularly in metastatic (Frenel et al. 2009; Opdam et al. 2012) and triple-negative tumours (T. Liu et al. 2011). Indeed, for the FMCp cell line, HER2-negative, an increase in 47.5% of cytotoxicity (from 6.9% to 54.4%) was obtained in the conjugation of lapatinib at 780 nM plus rapamycin at 6.25 nM, and by the use of neratinib at 3.125 nM plus rapamycin at 6.25 nM, where an increase of 44.1% of cytotoxicity (from 0.4% to 44.5%) was observed.

Beyond the referred acquired resistance to TKi, genomic mutations could also be responsible for a resistant profile to lapatinib, as well as neratinib. In breast cancer patients is known that *her2* is mutated in 2 to 3% of primary tumours and more than 70% of the mutations occurs in HER2-negative breast cancer subtype (Mishra et al. 2017), as in the FMC tumour tissue samples. In this study, a total of 42 different SVs were found in the feline *her2*-TK domain, with 90% (36/40) of the clinical samples showing at least one mutation. Furthermore, mutations revealed to be more common in intronic regions (54.8%; 23/42), particularly, between the exon 18 and 19 (60.9%), with 21.7% of them being identified in the splicing regions. The intron splicing locations revealed to be of extreme importance (Diederichs et al. 2016), as already reported in *her2* gene in cats (Santos et al. 2012) and in women, being associated with breast cancer risk (Høberg-Vetti et al. 2020) and responsible for resistance to therapy (Anczuków et al. 2012; Castagnoli et al. 2019). Regarding the exons, several mutations were found (45.2%; 19/42), with the majority of them showing a low frequency. Most of the mutations identified were located at the exon 18 (57.9%), being the heterozygous synonymous mutation c.19573 A>C associated to larger tumour sizes (p= 0.044) and occurring in luminal B and triple-negative carcinoma subtypes. Considering exon 20, which encodes for the location recognized by both TKi (Kong and Feldinger 2015; Sun et al. 2015), it comprises

36.8% of the mutations, in the FMC clinical samples. In the case of breast cancer patients, mutations within this exon were reported to increase the HER2 catalytic activity (Wang et al. 2006; Sun 2015), being the L755 (e.g., L755S and L755P), associated with resistance to lapatinib therapy (Kancha et al. 2011; Sun 2015; X. Xu et al. 2017; Collins et al. 2019), and L869R, together with T798I, promoting HER2 signalling and oncogenic growth, associated to neratinib acquired resistance (Hanker et al. 2017; Collins et al. 2019). Moreover, a rare mutation in breast tumours was identified in the feline luminal B and HER2-positive tumour subtypes, c.20385 T>G, that encodes for a STOP codon, leading to a truncated form of the protein and being associated with therapeutic resistance (Scott et al. 1993). Furthermore, the mutations described should not compromise the use of TKi in cats, since none of them were described as inducing resistance to therapy in breast cancer patients, according to National Cancer Institute, International Cancer Genome Consortium and COSMIC databases.

5. Conclusions

Beyond the cat being considered a noble breast cancer model, in this study it was possible to demonstrate valuable antiproliferative effects, and a conserved action mechanism of lapatinib and neratinib in three distinct feline mammary carcinoma cell lines, similarly to the results reported in the human breast cancer cell line (SkBR-3). Moreover, the obtained data suggests the use of FMC cell lines as *in vitro* tools for screening of new therapeutic drugs. In this study, the best results were achieved by the use of lapatinib, being possible to demonstrate a decrease in the phosphorylation pattern of the EGFR family members and its downstream pathways. Furthermore, combined assays with TKi and mTOR inhibitor showed synergistic effects, anticipating the benefits of using small drug doses. Since mutations found suggest that TKi-resistant FMC are very rare, altogether, our *in vitro* results strongly suggest the potential usefulness of TKi alone or combined with rapamycin in the treatment of cats with feline mammary carcinoma.

Author Contributions

A.G. and F.F. conceived and designed the experiments. A.G. performed the experiments. A.G., F.A., C.N., J.C. and F.F. analysed and interpreted the data. F.F. funding acquisition. F.F. methodology. F.F. supervision. F.F. project administration. A.G., F.F. Writing—Original draft. A.G., F.F., F.A., C.N., J.C. Writing—Review & editing. All authors contributed to and approved the final manuscript.

Acknowledgments

The authors would like to thank to Raffaella De Maria, University of Turin, Department of Veterinary Sciences, Italy that gave us all the feline cell lines (originally property from Nobuo Sasaki (DVM, PhD)

and Takayuki Nakagawa (DVM, PhD), from Graduate School of Frontier Science, University of Tokyo, Japan). We also would like to thank to Luís Costa, Molecular Medicine Institute, University of Lisbon, Portugal, that kindly ceded the human breast cancer cell line, SkBR-3.

Chapter III

Experimental Work

HER2-targeted immunotherapy and combined protocols showed promising antiproliferative effects in feline mammary carcinoma cell-based models

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Abstract

Feline mammary carcinoma (FMC) is a highly prevalent tumour, showing aggressive clinicopathological features, with HER2-positive being the most frequent subtype. While, in human breast cancer, the use of anti-HER2 monoclonal antibodies (mAbs) is common, acting by blocking the extracellular domain (ECD) of the HER2 protein and by inducing cell apoptosis, scarce information is available on use these immunoagents in FMC. Thus, the antiproliferative effects of two mAbs (trastuzumab and pertuzumab), of an antibody-drug conjugate compound (T-DM1) and of combined treatments with a tyrosine kinase inhibitor (lapatinib) were evaluated on three FMC cell lines (CAT-MT, FMCm and FMCp). In parallel, the DNA sequence of the *her2* ECD (subdomains II and IV) was analysed in 40 clinical samples of FMC, in order to identify mutations, which can lead to antibody resistance or be used as prognostic biomarkers. Results obtained revealed a strong antiproliferative effect in all feline cell lines, and a synergistic response was observed when combined therapies were performed. Additionally, the mutations found were not described as inducing resistance to therapy in breast cancer patients. Altogether, our results suggested that anti-HER2 mAbs could become useful in the treatment of FMC, particularly, if combined with lapatinib, since drug-resistance seems to be rare.

Keywords: Feline mammary carcinoma; HER2; monoclonal antibodies; combined therapies; tyrosine kinase inhibitors; feline *her2* mutations

1. Introduction

Similarly to human breast cancer (Soare and Soare 2019), the feline mammary carcinoma (FMC) is a very common tumour (Maria Soares, Ribeiro, et al. 2016), presenting different molecular subtypes (Maria Soares, Madeira, et al. 2016), being the feline HER2-positive, the most prevalent one (33 to 60%) (Soares et al. 2013; Maria Soares, Ribeiro, et al. 2016). The HER2-overexpression occurs associated to an AKT activation (Maniscalco et al. 2012), both markers of a poor prognosis and a high metastatic potential (Millanta et al. 2005; Maria Soares, Ribeiro, et al. 2016). In cats, a lack of therapeutic options lead most of the time to surgery (Michishita et al. 2016), being urgent the development of different therapeutic strategies, in order to improve the clinical outcome.

The epidermal growth factor receptor 2 (HER2) is a common target in patients with HER2-overexpression tumours (Appert-Collin et al. 2015). HER2 is a transmembrane glycoprotein, which modulates cell proliferation, differentiation and survival (Witton et al. 2003; Sun et al. 2015). This protein is composed by three domains: an extracellular domain (ECD), a short transmembrane region and an intracellular tyrosine kinase (TK) activity domain (Sun et al. 2015). Considering the ECD, it comprises four subdomains, I and II that are the membrane distal regions, and III and IV that are the membrane proximal regions, allowing protein-protein interaction and stabilization (Ferguson 2008; Sun et al. 2015). Since a sequence identity of 92% was reported between the human and feline *her2* (De Maria et al. 2005; Santos et al. 2013), the use of monoclonal antibodies (mAbs) in the feline mammary carcinoma, could be an alternative and attractive therapeutic approach.

Nowadays, the use of mAbs are widely common in HER2-positive breast cancer patients, decreasing its downstream pathways activation, such as AKT and mTOR, responsible for the anti-apoptotic mechanisms (Maniscalco et al. 2012), cell cycle progression and cell proliferation (Jhanwar-Uniyal et al. 2019), important checkpoints in the carcinogenesis process (Maniscalco et al. 2012). For example, trastuzumab (Piccart-Gebhart et al. 2005; Slamon et al. 2011; Oh and Bang 2020) is a monoclonal IgG antibody that inhibits HER2 homodimerization (Richard et al. 2016), blocking the HER2 pathway. This antibody prevents the receptor internalization and its degradation, stimulating antibody-dependent cellular cytotoxicity (ADCC) responses (Klapper et al. 2000) and promoting cell apoptosis (Hudis 2007; Valabrega et al. 2007). Trastuzumab resistance is documented in 40% of metastatic patients (Menyhart et al. 2015), due to changes in the HER2 expression status (Mittendorf et al. 2009) and structure (Scaltriti et al. 2007; Lipton et al. 2013), or other EGFRs pathway's activators (Kataoka et al. 2010; Jensen et al. 2012; Nami et al. 2018). In parallel, other mAb commonly used is pertuzumab, a recombinant IgG1 antibody anti-HER2 (Mullen et al. 2007; Oh and Bang 2020), valuable in combined therapeutic protocols (von Minckwitz et al. 2017). This mAb

inhibits the heterodimerization HER2-HER3 (Richard et al. 2016), inactivating the PI3K pathway and its downstream signalling cascades (Gerratana et al. 2017). This compound also stimulates ADCC (Nahta 2012), leading to cell-cycle arrest and apoptosis (Yamashita-Kashima et al. 2017). However, specific mutations can lead to pertuzumab resistance (Zhang et al. 2019; Gaibar et al. 2020). More recently, another compound in clinical use for breast cancer treatment is trastuzumab-emtansine (T-DM1) (von Minckwitz et al. 2019), which is an antibody-drug conjugate (ADC), consisting of the humanized monoclonal trastuzumab antibody covalently linked to the cytotoxic tubulin-binding agent (DM1) (Phillips et al. 2008). T-DM1 mechanism is associated to trastuzumab-HER2 conjugation and the release of the DM1 molecule after complex degradation into lysosomes (Klute et al. 2014). The efficacy of this drug depends on the cell membrane HER2 concentration (Barok et al. 2014) and allows to decrease the systemic cytotoxic effects of DM1, by its specific delivery to the tumour HER2-overexpression cells (Barok et al. 2014; Klute et al. 2014), triggering autophagy and apoptosis (Liu et al. 2020). T-DM1 leads to the inhibition of HER2 ECD shedding and PI3K/AKT pathway, stimulates ADCC, mitotic arrest and disruption of the intracellular trafficking. Also for this compound, several different mechanisms of resistance are described, not only associated with the HER2 protein, as well as STAT3 activation (Wang et al. 2018), defective cyclin B1 induction (Sabbaghi et al. 2017) and multidrug resistance proteins (Barok et al. 2014).

In human breast cancer, 50% of the HER2-positive patients show immunotherapy resistance (Schroeder et al. 2014), being the combined therapeutic protocols truly valuable (Tóth et al. 2016). As frequently used trastuzumab plus pertuzumab (Nahta et al. 2004; Richard et al. 2016; Tóth et al. 2016; Nami et al. 2018) leads to a synergistic response and increased ADCC effects (Harbeck et al. 2013; Gerratana et al. 2017). Other common combination is the use of mAbs with tyrosine kinase inhibitors (TKi), which are small molecules that binds to the cytoplasmic catalytic kinase domain of the HER2, preventing tyrosine phosphorylation and signalling (Schroeder et al. 2014), such as lapatinib (Okita et al. 2015; Watson et al. 2018; Canonici et al. 2019).

An early diagnosis and an individual therapy is crucial for the improvement of survival time (Cheung 2020) and prevention of therapy resistance in cats with mammary carcinoma (Schroeder et al. 2014). Thus, this study aims to: 1) evaluate the antiproliferative effects of the mAbs (trastuzumab and pertuzumab) and of the T-DM1 in three feline carcinoma cell lines (CAT-MT, FMCm and FMCp); 2) characterize the HER2 expression in the feline cell lines and identify the existence of genomic mutations in the feline *her2* ECD (subdomains II and IV); 3) describe the cell death mechanism induced by the mAbs and T-DM1, in the carcinoma cell lines; 4) evaluate the synergistic antiproliferative effects by the combination of mAbs (trastuzumab plus pertuzumab) and assess the increase in the cytotoxic response by the use of mAbs with the TKi (lapatinib); and 5) identify genomic mutations in the feline *her2* gene ECD

(subdomains II and IV) in FMC clinical samples, in order to recognize possible therapy resistant animals, or prognostic factors.

2. Materials and Methods

2.1. Feline and human mammary carcinoma cell lines

In this study, three feline cell lines (CAT-MT from the European Collection of Authenticated Cell Culture, England; FMCp and FMCm kindly provided by Prof. Nobuo Sasaki and Prof. Takayuki Nakagawa, University of Tokyo, Japan) (Maniscalco et al. 2012; Maniscalco et al. 2013) and a positive control, the HER2-overexpressing human breast cancer cell line (SkBR-3 from the American Type Culture Collection, Manassas, VA, USA) were used, after characterized by immunocytochemistry (**Figure S2.1 – Annex II** and **Table S2.1 – Annex II**). Cell cultures were maintained at 37°C, in a humidified atmosphere of 5% (v/v) CO_2 (Nuaire, Plymouth, MN, USA), in Dulbecco's Modified Eagle Medium (DMEM; Corning, New York, NY, USA), for CAT-MT and SkBR-3, whereas FMCm and FMCp cell lines were maintained in Roswell Park Memorial Institute 1640 Medium (RPMI; Corning), both supplemented with heat-inactivated 20% (v/v) fetal bovine serum (FBS; Corning). Periodically, all cell lines were inspected to control their morphology and proliferation rate, being tested for mycoplasma (MycoSEQ™ Mycoplasma Detection Kit, Thermo Fischer Scientific, Waltham, MA, USA).

2.2. In vitro cytotoxicity assays

Viability assays were performed to determine the antiproliferative effects of trastuzumab, pertuzumab and T-DM1 (all from Roche, Basel, Switzerland), using the Cell Proliferation Reagent WST-1 (Abcam, Cambridge, United Kingdom) and following the manufacturer's instructions. Briefly, cell lines were seeded in 96-well plates to obtain a confluency of 90%, after 24 hours ($5x10^3$ cells/well for CAT-MT and FMCp, $15x10^3$ cells/well for FMCm and $10x10^3$ cells/wells for SkBR-3), and then exposed to increasing concentrations of each antibody (**Table 6**), with the control wells left unexposed. Phosphate buffered saline (PBS; Corning) was used as a vehicle for mAbs and ADC. After 72 hours of exposure, the WST-1 reagent (Abcam) was added, followed by an incubation period of 4 hours, at 37°C, and absorbance was measured at 440 nm using a plate reader (FLUOStar Optima, BMG LabTech, GmbH, Ortenberg, Germany). Triplicate wells were used to determine each data point and three independent experiments were performed.

Table 6. Concentrations of trastuzumab (µg/mL), pertuzumab (µg/mL) and T-DM1 (µg/mL) used in the cytotoxicity assays. Cells were exposed to drugs for 72 hours and the antiproliferative effects were evaluated.

Drug concentrations for the cytotoxicity assays (µg/mL)		
Trastuzumab	**Pertuzumab**	**T-DM1**

25	50	5
50	100	12.5
125	250	25
250	500	50
500	1000	100
1000	2000	200
2000	5000	500
5000	10,000	1000
10,000		

For the combined assays: trastuzumab plus pertuzumab, trastuzumab plus lapatinib (Sigma-Aldrich, Darmstadt, Germany) and pertuzumab plus lapatinib, a similar methodology was used, testing concentrations that covers different cytotoxic responses (**Table 7**).

Table 7. Concentrations of trastuzumab (µg/mL), pertuzumab (µg/mL) and lapatinib (µg/mL) used in the combined treatments. Cells were exposed to drugs for 72 hours before cytotoxicity evaluation.

Drug concentrations for the combined treatments (µg/mL)		
Trastuzumab	**Pertuzumab**	**Lapatinib**
125	250	0.453
500	2000	7.26

2.3. Assessment of HER2 expression status by immunocytochemistry (ICC)

Expression analysis of HER2 was performed, as reported by us (Soares et al. 2013; Almeida et al. 2021), in the three feline cell lines (CAT-MT, FMCm and FMCp), and using the human SkBR-3 cell line as a positive control. Briefly, cells grown till confluency in a T25 culture flask and then were removed and embedded in a histogel matrix (Thermo Fischer Scientific). Cytoblocks were sectioned in slices with 3 µm thickness (Microtome Leica RM135, Newcastle, UK) and mounted on a glass slide (SuperFrost Plus, Thermo Fisher Scientific). On PT-Link module (Dako, Agilent, Santa Clara, CA, USA) samples were deparaffinised, hydrated and antigen retrieval was performed for 20 minutes at 96°C, using citrate buffer pH 6.1 (EnVision™ Flex Target Retrieval Solution Low pH, Dako). Then, slides were cooled for 30 minutes at room temperature (RT) and immersed twice in distilled water for 5 minutes. ICC technique was performed with commercial solutions from the EnVision™ FLEX+, Mouse kit (Dako). Before antibody incubation, samples were treated with Peroxidase Block Novocastra Solution (Leica Biosystems) for 15 minutes. Afterwards, samples were incubated with a primary antibody anti-HER2 (clone CB11, 1:100, ab8054; Abcam) by 1 hour at RT, in a humidified chamber. Then, the EnVision™ FLEX+ Mouse Linker was incubated by 30 minutes and slides were washed for 5 minutes, between all the incubation steps, using PBS at pH 7.4. Later on, the EnVision™ FLEX/HRP was incubated for 30 minutes at RT, and detection was performed using

diaminobenzidine (DAB substrate buffer and DAB Chromogen, Dako) for 5 minutes. Finally, samples were counterstained with Gill's haematoxylin (Merck, Darmstadt, Germany) for 5 minutes, dehydrated in an ethanol gradient and xylene, and mounted using Entellan mounting medium (Merck).

HER2 immunoreactivity was scored as recommended by the American Society of Clinical Oncology's (ASCO) (Wolff et al. 2013), and as previously published for feline cells (Soares et al. 2013; Maria Soares, Ribeiro, et al. 2016). Briefly, the staining intensity was evaluated and classified as HER2-negative when scored 0 and HER2-positive if scored 1+, 2+ or 3+ (**Table 8**). Three microscopic fields were analysed at 400x magnification. All samples were subjected to a blind scoring, by two independent pathologists.

Table 8. HER2 immunocytochemistry scoring criteria.

Score	Interpretation
0	No staining
1+	Weak, incomplete membrane staining
2+	Complete membrane staining, with obvious circumferential distribution in at least 10% of cells, that has either no uniform or is weak in intensity
3+	Uniform and intense membrane staining, at a minimum of 10% of the tumour cells

2.4. Flow cytometry assay

The flow cytometry assay was performed as already reported by us (Almeida et al. 2021). For this experiment, the cells were seeded in 24-well plates to obtain a confluency of 90% after 24 hours ($5x10^4$ cells/well for CAT-MT and FMCp, $10x10^4$ cells/well for FMCm, and $7x10^4$ cells/well for SkBR-3 cell lines), and then were exposed to mAbs and to the ADC for 72 hours, at a concentration close to the EC_{50} value. Control wells were left unexposed, with the PBS used as a vehicle, in three independent experiments. The percentage of apoptotic cells after drug exposure was calculated by using the APC Annexin V Apoptosis Detection Kit with Propidium Iodide (PI; BioLegend, San Diego, CA, USA) and following the manufacturer's instructions. Briefly, supernatants were harvested and the remaining attached cells were trypsinized (Trypsin-EDTA; Corning) and added to the correspondent supernatants. Then, samples were centrifuged for 5 minutes at 500 g at RT, washed with PBS and resuspended in 500 µL of Annexin V Binding Buffer (BioLegend), with a maximum concentration of $1x10^7$ cells/mL. Afterwards, 100 µL of each sample were passed to a new tube, and added 5 µL of APC Annexin V (BioLegend) and 10 µL of PI (BioLegend). Then, samples were vortexed and incubated by 15 minutes at RT, protected from the light. Finally, 400 µL of Annexin V Binding Buffer (BioLegend) were added to the samples, before acquisition in a BD LSR Fortessa X-20

(BD Biosciences, San Jose, CA, USA), at Champalimaud Foundation, Lisbon, Portugal. Data were analysed using FlowJo software (v.10.7.1, for Windows, BD Biosciences), considering double negative staining cells, as living cells, annexin positive cells in early apoptosis and double positive cells (both for annexin and PI) in late apoptosis phase.

2.5. Animal population

The forty tumour tissue samples used in this study were collected from cats that underwent mastectomy, at the Teaching Hospital of the Faculty of Veterinary Medicine, University of Lisbon, with all the procedures consented by the owners, showing no interference in the animals' well-being. The clinical history of the cats was recorded (**Table 9**), including breed, age, reproductive status and contraceptive administration, treatment (mastectomy or mastectomy plus chemotherapy), number, location and size of tumour lesions, histopathological classification, ER status, PR status, HER2 status and Ki-67 index, malignancy grade, tumour necrosis, lymphatic invasion, lymphocytic infiltration, cutaneous ulceration, regional lymph node involvement and clinical stage (TNM system) (Maria Soares, Ribeiro, et al. 2016), with tumours being classified into five molecular subtypes (Maria Soares, Madeira, et al. 2016). Additionally, the *f*HER2 was determined has being overexpressed in 33% of the FMC cases, despite *her2* gene amplification was not observed (Soares et al. 2013). All samples were frozen at -80ºC and stored until further use.

Table 9. Clinicopathological features of female cats with mammary carcinomas enrolled in this study (n= 40).

Clinicopathological feature	Number (%)	Clinicopathological feature	Number (%)
Breed		**Age**	
Indeterminate	33 (82.5%)	<8years old	3 (7.5%)
Siamese	4 (10%)	≥8 years old	37 (92.5%)
Persian	2 (5%)	**Tumour Size**	
Norwegian Forest	1 (2.5%)		
		<2cm	9 (22.5%)
Spayed; 1 unknown		2-3cm	19 (47.5%)
Yes	19 (47.5%)	>3cm	12 (30%)
No	20 (50%)	**HP* classification**	
Contraceptives; 7 unknown		Tubulopapillary carcinoma	8 (20%)
Yes	23 (57.5%)	Solid carcinoma	9 (22.5%)
No	10 (25%)	Cribiform carcinoma	5 (12.5%)
Treatment		Mucinous carcinoma	5 (12.5%)
Mastectomy	36 (90%)	Tubular Carcinoma	11 (27.5%)
Mastectomy+Chemo	4 (10%)	Papillary-cystic carcinoma	2 (5%)
Multiple tumours		**HP* Malignancy grade**	
Yes	31 (77.5%)	I	2 (5%)
No	9 (22.5%)	II	5 (12.5%)

Regional Lymph node status; 2 unknown		III	33 (82.5%)
Positive	14 (35%)	**Tumour Necrosis**	
Negative	24 (60%)	Yes	29 (72.5%)
Stage (TNM classification)		No	11 (27.5%)
I	9 (22.5%)	**Lymphatic invasion**	
II	7 (17.5%)	Yes	5 (12.5%)
III	21 (52.5%)	No	35 (87.5%)
IV	3 (7.5%)	**Lymphocytic infiltration**	
Mammary location		Yes	27 (67.5%)
M1	11 (27.5%)	No	13 (32.5%)
M2	8 (20%)	**Tumour ulceration**	
M3	14 (35%)	Yes	3 (7.5%)
M4	11 (27.5%)	No	37 (92.5%)
fHER2 status		**Ki-67 index**	
Positive	12 (30%)	Low (<14%)	30 (75%)
Negative	28 (70%)	High (≥14%)	10 (25%)
ER status		**PR status**	
Positive	12 (30%)	Positive	20 (50%)
Negative	28 (70%)	Negative	20 (50%)
Tumour molecular subtype			
Luminal A	3 (7.5%)		
Luminal B	18 (45%)		
Luminal B/HER2-positive	8 (20%)		
HER2-positive	4 (10%)		
Triple-negative	7 (17.5%)		

*HP – Histopathological; TNM – Tumour, Node, Metastasis; ER – Oestrogen receptor; PR – Progesterone receptor

2.6. DNA extraction, amplification and sequence analysis of feline her2 ECD

Genomic DNA extraction was performed in 5 mg of 44 frozen tissue samples (4 breed control and 40 tumour tissues) and in the three feline cell lines (CAT-MT, FMCm and FMCp), collected after grown till confluency in a T25 culture flask, as previously described (Santos et al. 2009; Ferreira, Soares, et al. 2019), using a QIAmp FFPE kit (Qiagen, Dusseldorf, Germany) and following the manufacturer's guidelines. First, tissue samples were homogenized in the Tissue Lyser II (Qiagen), and then, all samples were digested with protease K (20 mg/mL; Qiagen). After the washing steps, genomic DNA was eluted from the extraction columns and its quality and quantity was measured in NanoDrop ND-100 Spectrophotometer (Thermo Fischer Scientific). For the amplification of exons 3, 4 and 10 to 13, which encode for the feline *her2* ECD (NC_018736.3), previously identified by comparison with the genomic human *her2* sequence (NC_000017.11), different primers were designed (**Table 10**) in the Primer designing tool (NCBI, Bethesda, MD, USA), and used in a PCR Thermal Cycler (VWR Thermocycler, Leicestershire, England). PCR technique was performed

with a standard reaction mixture [4 µL/sample of Phusion GC buffer (Thermo Fischer Scientific), 0.4 µL/sample of dNTPs (Grisp, Porto, Portugal), 0.1 µL/sample of each forward and reverse primers and 0.2 µL/sample of DNA Polymerase (Thermo Fischer Scientific)], maintaining a final DNA concentration of 4 ng/mL. For exons 3, 4, 10 and 11, PCR reactions were performed as follows: denaturation at 98°C for 30 seconds, followed by 35 cycles at 98°C for 10 seconds, 58°C for 30 seconds, 72°C for 10 seconds, plus one final extension step at 72°C for 10 minutes. For exons 12 and 13 a nested-PCR was performed, using two pairs of primers, initially with a melting temperature of 55°C, followed by a melting temperature of 52°C for the second pair of primers. After confirmation of the expected size for each amplified sequence in a 2% agarose gel (Sigma-Aldrich), DNA fragments were purified and sequenced by Sanger technique (StabVida, Almada, Portugal), and checked for inaccuracies.

Table 10. Primers for genomic DNA amplification and sequencing of exons 3, 4 and 10 to 13 of the feline *her2* ECD.

Exons	Forward (5´-3´)	Reverse (5´-3´)
3	GGCGCTTGCTCATAGTTCAC	ATCAAACTGTGCAGGCTCGT
4	GAGGCCTGCTCCCCTCTAAA	AAGAGGGAATGGGTAGCGTT
10 – 11	GGGCTTGGGCTTTGAAACTC	TGAAGGGTCAGCGAGTAAGC
12 – 13 (1st pair)	TGGGAGTTTTCGGAGTGTGC	AAGCCTGACAGAAGGGATGG
12 – 13 (2nd pair)	GTGCTTACTCGCTGACCCTTCA	ACCCCTGCAATACTCGGCATTC

The feline sequenced samples were aligned with the identified feline *her2* (NC_018736.3), using the ClustalW tool (BioEdit Alignment Editor software) (Hall 1999), while the consensus sequence was confirmed using the SeqTrace 9.1 software (Stucky 2012). Protein mutations and single nucleotide polymorphism (SNP) loci were identified using the Expert Protein Analysis System (ExPASY) translate tool and compared with the original protein sequence (NP_001041628.1, NCBI). Mutations identified in the feline tissue samples were compared to the human *her2* sequence (NC_000017.11) and searched in National Cancer Institute, International Cancer Genome Consortium and Catalogue of Somatic Mutations in Cancer (COSMIC) databases for putatively induced resistance to mAbs, and the ADC tested in this study.

2.7. Statistical analysis

Statistical analysis was carried out using the GraphPad Prism software (version 5.04, for Windows, San Diego, CA, USA), with a two-tailed *p*-values below 0.05 being considered statistically significant, and a 95% confidence interval (**p*< 0.05, ***p*< 0.01 and ****p*< 0.001). For the cytotoxicity assays, outliers with more than two standard deviations were removed from the analysis and the EC_{50} value for each drug, in all the cell lines was calculated using Log (agonist) *vs.* Response (Variable slope) function. In the drug conjugation assays, the two-way

ANOVA test was performed. Regarding the mutations found in tissue samples, associations between groups were assessed using the non-parametric Mann Whitney test.

3. Results

3.1. Trastuzumab, pertuzumab and T-DM1 presented antiproliferative effects in FMC cell lines

Trastuzumab, pertuzumab and T-DM1 exhibited a dose-dependent antiproliferative effect on feline mammary carcinoma cell lines. Indeed, trastuzumab exerted a more potent antiproliferative effect on CAT-MT cell line (92.6% of cytotoxicity, EC_{50}= 3047.89 µg/mL±1.43; **Figure 12A**) in comparison with the effects on the FMCm (82.7% of cytotoxicity, EC_{50}= 528.45 µg/mL±1.14) and FMCp (60.1% of cytotoxicity, EC_{50}= 3243.40 µg/mL±2.29) cell lines. In parallel, pertuzumab showed similar results on CAT-MT and FMCm cell lines (60.2% and 61.8% of cytotoxicity, EC_{50}= 2837.92 µg/mL±1.50 and EC_{50}= 1205.04 µg/mL±1.23, respectively; **Figure 12B**), with the feline HER2-negative cell line FMCp, presenting the lowest antiproliferative effect (52.1% of cytotoxicity, EC_{50}= 928.97 µg/mL±1.11). Regarding the ADC T-DM1, the highest antiproliferative effect, at the range of concentrations tested, was reported on CAT-MT and FMCp cells (94.0% and 74.2% of cytotoxicity, EC_{50}= 19.63 µg/mL±1.22 and EC_{50}= 88.72 µg/mL±1.29, respectively; **Figure 12C**), with similar cytotoxicity being detected on FMCm and SkBR-3 cell lines (53.8% and 50.5% of cytotoxicity, respectively; EC_{50} (FMCm)= 52.84 µg/mL±1.50).

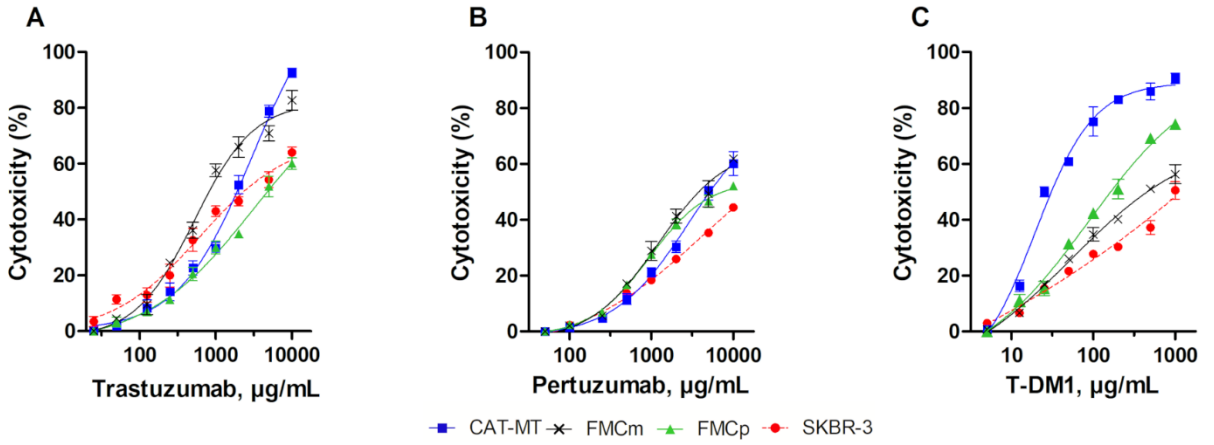

Figure 12. Trastuzumab, pertuzumab and T-DM1 presented strong antiproliferative effects on feline mammary carcinoma cell lines. **A)** CAT-MT cells exposure to trastuzumab allowed to obtain a maximum of 92.6% of cytotoxicity. **B)** Cell lines exposure to pertuzumab presented a lower antiproliferative effect, with a maximum of 61.8% of cytotoxicity in the FMCm cells. **C)** T-DM1 had the highest antiproliferative effect in the CAT-MT cells, with 94.0% of cytotoxicity. The experiments were performed in triplicates, in three independent assays.

In order to interpret the response of cell lines to anti-HER2 agents, the HER2 expression status was determined. Interestingly, all feline cell lines showed lower HER2 scores than the human SkBR-3 cell line (scored as 3+, **Figure 13D**), with CAT-MT cells showing a 2+

score (**Figure 13A**), FMCm cells showing 1+ HER-2 positivity (**Figure 13B**) and FMCp cells showing no immunostaining signal (0 score, **Figure 13C**).

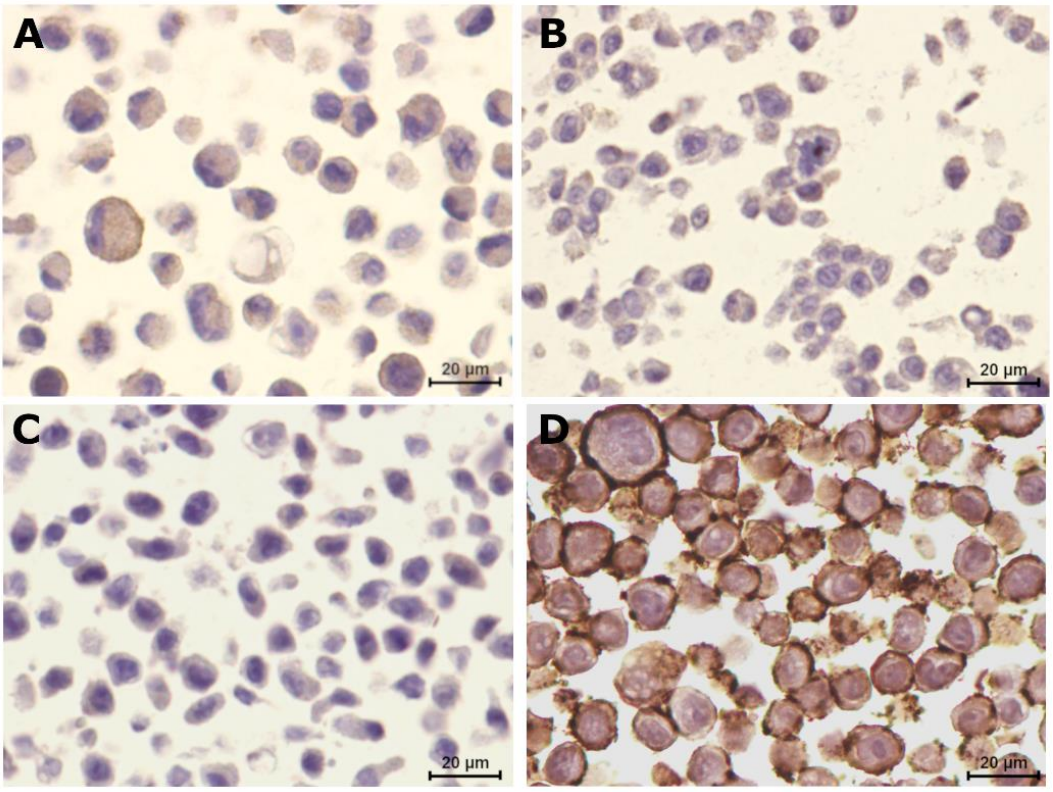

Figure 13. Feline mammary carcinoma cell lines presented different HER2 expressions. **A)** CAT-MT cell line was classified as HER2-positive (HER2 2+ score). **B)** FMCm was classified with 1+ score, being considered slightly positive for HER2 expression. **C)** FMCp cell line presented no HER2 signal (HER2-negative). **D)** The human SkBR-3 cell line was classified as a HER2-overexpressing cell line (3+ score). (400x magnification)

3.2. Apoptosis is the main mechanism of cell death caused by anti-HER2 mAbs and ADC T-DM1

Flow cytometry analysis of feline cell lines exposed to anti-HER2 mAbs (trastuzumab and pertuzumab) or ADC (T-DM1) allowed to confirm that the observed antiproliferative effects were related to apoptosis induction (**Figure 14**). Deepening the analysis, we observed that the HER2-overexpressing CAT-MT cell line showed the higher percentage of cells in late apoptosis for both mAbs, reaching a maximum of 53.1%±2.54 of apoptotic cells after trastuzumab exposure (14.1% early apoptosis and 39.0% late apoptosis) and 65.3%±2.33 after treated with pertuzumab (12.8% early apoptosis and 52.5% late apoptosis; **Figure 14A**). The balance between early and late apoptosis was similar when cells were treated with T-DM1 (41.7% and 39.6%, respectively), with a maximum of 81.3%±1.61 of apoptotic cells. In FMCm cell line the percentage of cells in late apoptosis was the highest, presenting 52%±4.02 of apoptotic cells (10.5% early apoptosis and 41.5% late apoptosis) after exposure to trastuzumab, 44.4%±1.04 as response to pertuzumab (12.1% early apoptosis and 32.3% late apoptosis) and 74.2%±6.06 of apoptotic cells (25.1% early apoptosis and 49.1% late

apoptosis) after T-DM1 incubation (**Figure 14B**). A different behaviour was observed in the FMCp cell line that showed a higher percentage of cells in early apoptosis. The total percentage of apoptotic cells after exposure to trastuzumab was 44.5%±1.64 (36.4% early apoptosis and 8.1% late apoptosis), and 52.3%±1.79 (47.1% early apoptosis and 5.2% late apoptosis) after pertuzumab exposure. In these cells, T-DM1 induced 44.5%±2.63 of cellular apoptosis (41.9% early apoptosis and 2.6% late apoptosis; **Figure 14C**). Lastly, the human SkBR-3 cells used as control, showed expected results, with an average of 48.8%±1.62 of apoptotic cells when exposed to trastuzumab and pertuzumab, similarly distributed between early and late apoptosis stages (average 22.2% and 26.6%, respectively), with the percentage of apoptotic cells being higher after T-DM1 exposure (80.1%±2.00; 27.5% early apoptosis and 52.6% late apoptosis; **Figure 14D**).

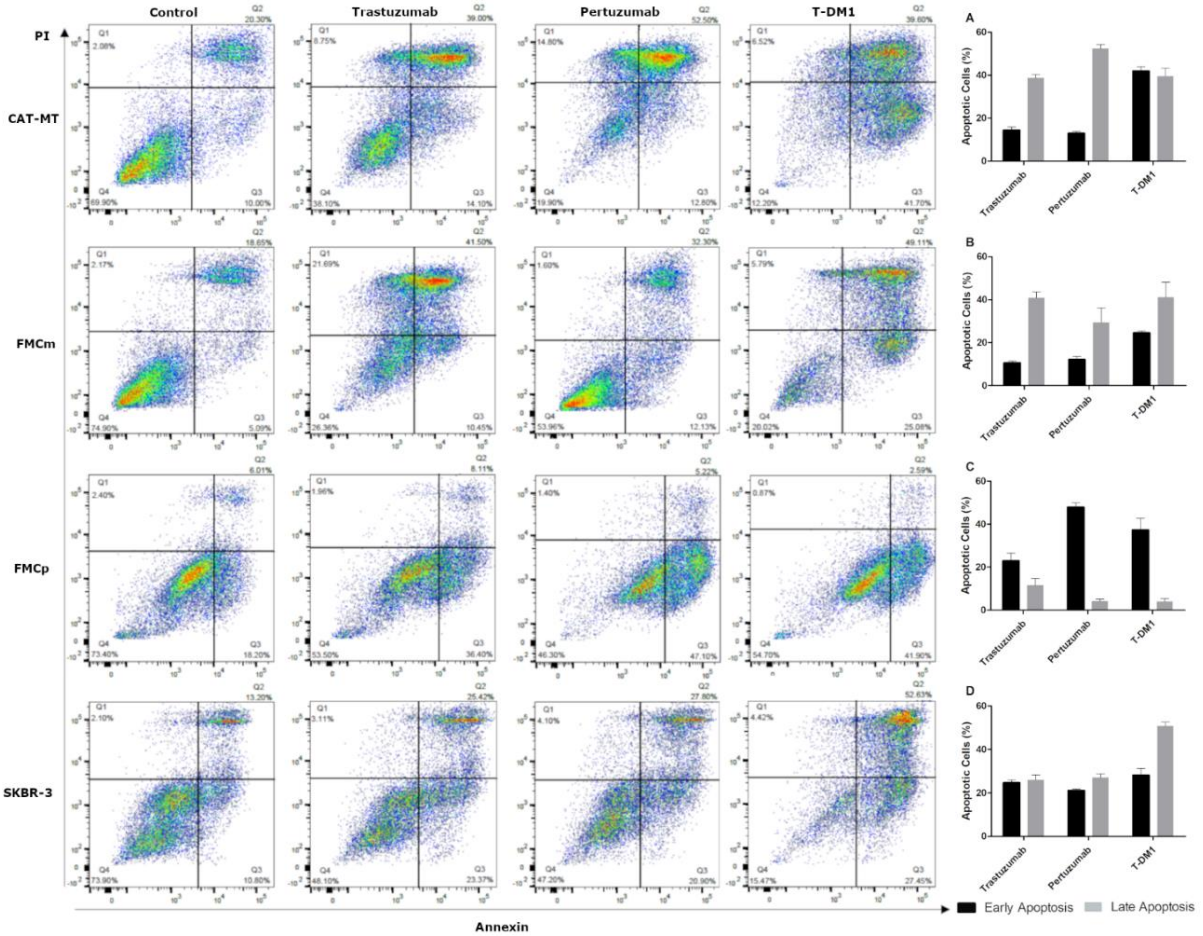

Figure 14. Trastuzumab, pertuzumab and T-DM1 induce apoptosis in the feline cell lines. **A)** CAT-MT and **B)** FMCm cell lines presented an increase in cell apoptosis after drugs exposure, being the cells predominantly in late apoptosis phase. **C)** In the FMCp cell line, cells occurred predominantly in early stage apoptosis. **D)** The human SkBR-3 cell line had an equilibrated death pattern, between early and late stage apoptosis, with the cells mainly in late apoptosis phase, by exposure to T-DM1. The experiment was performed in triplicates and repeated simultaneously in three times.

3.3. Combined exposures of two anti-HER2 mAbs and anti-HER2 mAbs with lapatinib showed synergistic antiproliferative effects

The combination trastuzumab plus pertuzumab showed synergistic antiproliferative effects in all feline cell lines. In CAT-MT cells the exposure to trastuzumab at 125 µg/mL plus pertuzumab at 2000 µg/mL increased the cytotoxicity from 13.6% to 40.0% (p< 0.001; **Figure 15A**). For the FMCm cells, the cytotoxic effect was increased from 15.6% to 45.1% (p< 0.001; **Figure 15B**), after exposure to trastuzumab at 125 µg/mL plus pertuzumab at 2000 µg/mL. Finally, in the FMCp cell line, the highest antiproliferative effect was obtained using trastuzumab at 500 µg/mL plus pertuzumab at 2000 µg/mL, increasing the cytotoxicity from 17.1% to 28.8% (p= 0.018; **Figure 15C**). In the human SkBR-3 cells, all the conjugations tested exerted a significant increase of the antiproliferative effect (p< 0.001; **Figure 15D**).

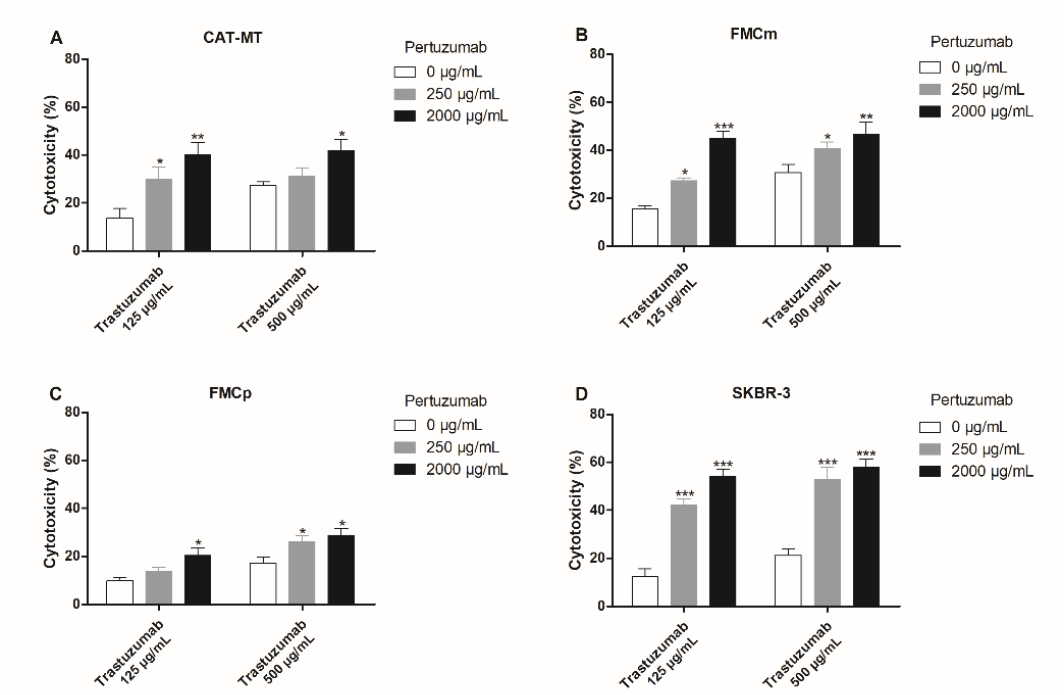

Figure 15. Combined treatments with trastuzumab plus pertuzumab showed synergistic antiproliferative effects in the carcinoma cell lines. **A)** CAT-MT cells presented the highest cytotoxic response by the use of trastuzumab at 125 µg/mL plus pertuzumab at 2000 µg/mL, being possible to achieve 40.0% of cytotoxicity (**p< 0.01). **B)** In FMCm cell line, the combination trastuzumab at 125 µg/mL plus pertuzumab at 2000 µg/mL was the one that allows to achieve the highest cytotoxic effect, 45.1% of cytotoxicity (***p< 0.001). **C)** In the FMCp cells a maximum cytotoxic effect of 28.8% using the combination trastuzumab at 500 µg/mL plus pertuzumab at 2000 µg/mL (*p< 0.05) was achieved. **D)** Similarly to the feline cell lines, in the human SkBR-3 cell line synergistic antiproliferative effects were obtained, revealing all the combinations valuable. The experiments were performed in triplicates and repeated three separated times.

The use of the anti-HER2 mAbs (trastuzumab and pertuzumab) combined with lapatinib showed an improvement of the antiproliferative effects induced by mAbs. Accordingly, the combination of trastuzumab plus lapatinib, in the CAT-MT cell line, revealed a valuable synergistic response, for all the concentrations tested, with the highest increase of 78.4% of

cytotoxicity, by the combination of trastuzumab at 125 µg/mL plus lapatinib at 7.26 µg/mL (p< 0.001; **Figure 16A**). The other two feline cell lines (FMCm and FMCp) presented different behaviours. While, the FMCm cells presented a lower response, with the highest antiproliferative effect occurring by exposure to trastuzumab at 125 µg/mL plus lapatinib at 7.26 µg/mL, increasing the cytotoxicity from 10.2% to 37.4% (p= 0.0017; **Figure 16B**), the FMCp cells had a significant decrease in its proliferation rate for all the combinations tested (p< 0.001; **Figure 16C**), with a maximum cytotoxic effect of 84.6% of cytotoxicity, by combining trastuzumab at 500 µg/mL plus lapatinib at 7.26 µg/mL. In the SkBR-3 cell line all the concentrations tested presented an effective synergistic response (p< 0.001; **Figure 16D**).

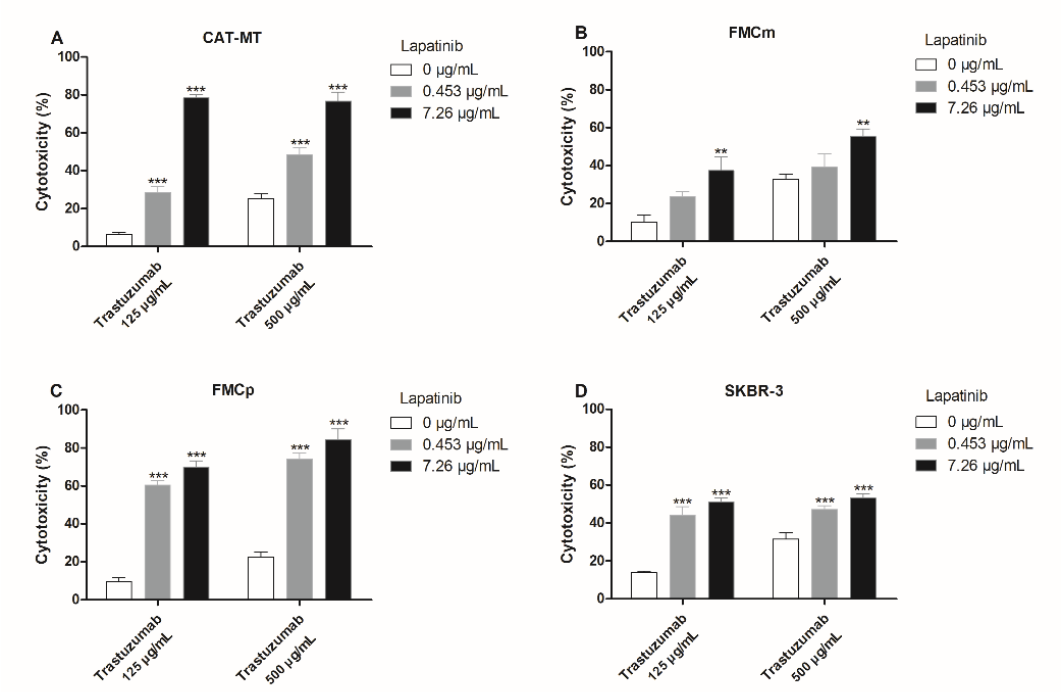

Figure 16. Combined treatments with trastuzumab plus lapatinib allowed to obtain a valuable synergistic antiproliferative effect in the carcinoma cell lines. **A)** CAT-MT and **C)** FMCp cells presented a significant increase in the cytotoxicity for all the concentrations tested (***p< 0.001). **B)** FMCm cells presented the highest increase in the cytotoxic effect by the combination of trastuzumab at 125 µg/ml plus lapatinib at 7.26 µg/mL, being possible to achieve 37.4% of cytotoxicity (**p< 0.01). **D)** In the human SkBR-3 cell line, all the combinations presented a synergistic antiproliferative effect (***p< 0.001). The experiments were performed in triplicates and repeated three separated times.

Similarly to the previous results, the combination of pertuzumab plus lapatinib also revealed to be valuable, increasing the mAb's antiproliferative effects. In fact, the combination of pertuzumab at 250 µg/mL plus lapatinib at 7.26 µg/mL, in the CAT-MT cells revealed a synergistic effect, with an increase from 7.4% to 76.8% of cytotoxicity (p< 0.001; **Figure 17A**). Again, the FMCm cell line presented to be the less sensitive, being obtained a maximum antiproliferative effect by exposure to pertuzumab at 250 µg/mL plus lapatinib at 7.26 µg/mL, increasing the cytotoxicity from 6.3% to 47.8% (p< 0.001; **Figure 17B**). In the FMCp cells the highest increase in the cytotoxic effect was observed using pertuzumab at 250 µg/mL plus

lapatinib at 7.26 µg/mL, increasing the cytotoxicity from 6.2% to 53.7% (p< 0.001; **Figure 17C**). As control, the human cell line SkBR-3 also presented an effective increase in the antiproliferative effects of the combined assays (p< 0.001; **Figure 17D**), for all the concentrations tested.

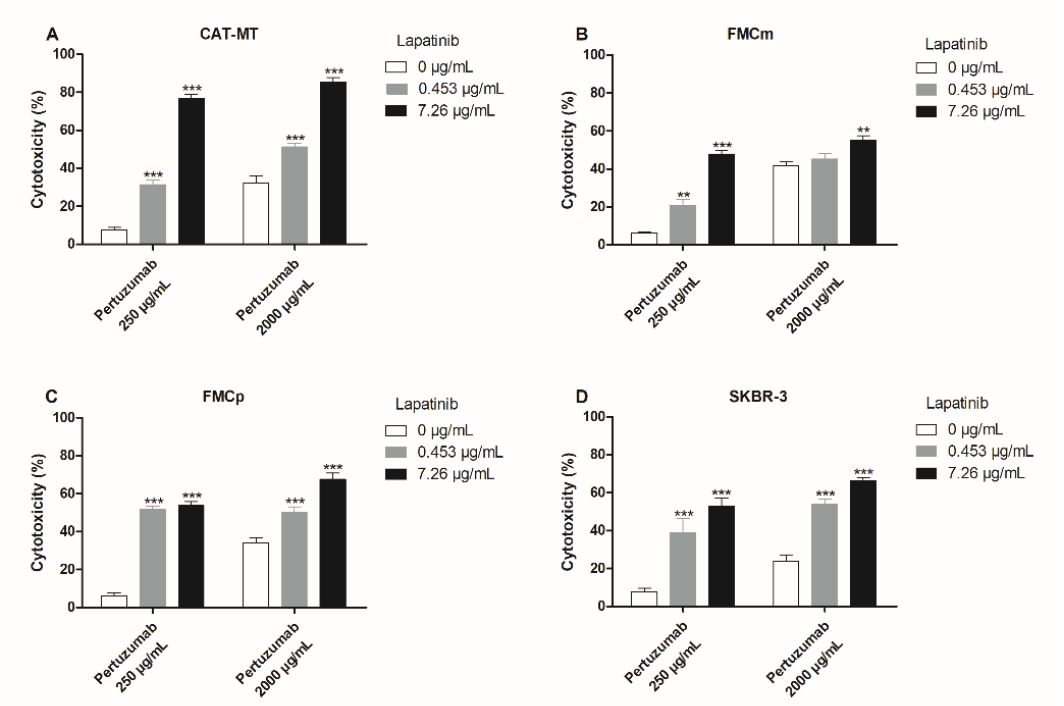

Figure 17. The combination of pertuzumab with lapatinib showed an improved antiproliferative effect in all the carcinoma cell lines. **A)** CAT-MT cells presented synergistic antiproliferative effects for all the conjugations tested, occurring the highest cytotoxic response, by the use of pertuzumab at 250 µg/mL plus lapatinib at 7.26 µg/mL, with 76.8% of cytotoxicity (***p< 0.001). **B)** The FMCm cells achieved the highest synergist effect, by the use of pertuzumab at 250 µg/mL plus lapatinib at 7.26 µg/mL, with 47.8% of cytotoxicity (***p< 0.001), while the combination of pertuzumab at 2000 µg/mL plus lapatinib at 7.26 µg/mL presented a smaller cytotoxic effect (**p< 0.01). **C)** The combination of pertuzumab at 250 µg/mL plus lapatinib at 7.26 µg/mL, in the FMCp cells, allowed to obtain 53.7% of cytotoxicity (***p< 0.001). **D)** As control, the human SkBR-3 cell line presented a synergistic effect for all the conjugations tested (***p< 0.001). The experiments were performed in triplicates and repeated three separated times.

3.4. In the FMC clinical samples, mutations found in the her2 ECD, subdomains II and IV were not associated with immunotherapy resistance in humans

Previous studies on somatic mutations in the *her2* gene, in human breast cancer patients, revealed several mutations associated with resistance to therapy and/or specific clinicopathological features, reported in National Cancer Institute, International Cancer Genome Consortium and COSMIC databases. In this work, the FMC clinical samples revealed that, 45% (18/40) presented, at least, one mutation (**Figure 18**; **Table S2.2 – Annex II**), with all of them showing a low frequency (n= 1), and its majority being homozygous (83.3%; 30/36 mutations). Detected mutations were more frequently in luminal B tumours (61.1%; 11/18), followed by the luminal B/HER2-positive subtype (50%; 4/8) and triple-negative tumour samples (42.9%; 3/7), with no mutations identified in HER2-positive (n= 4) and luminal A (n=

3) tumour subtypes. Further analysis revealed that in the region recognized by pertuzumab, *her2* subdomain II, while 69.4% (25/36 mutations) of the mutations were located in exon 3 (11/40 tumour samples), no mutations were found in the exon 4. Additionally, exon 3 presented 11 synonymous and 10 missense mutations, being the remaining 4, silent mutations. Regarding the subdomain IV, which is the *her2* region recognized by trastuzumab, few mutations were detected, namely 1 heterozygous missense mutation at exon 10 (2.8%; 1/36), in 2 tumour samples; 5 mutations in exon 11 (13.9%; 5/36 mutations), being 1 heterozygous synonymous mutation and 4 missense mutations, occurring in 3 tumour samples; 2 mutations at exon 12 (5.6%; 2/36 mutations), occurring in 2 tumour samples, 1 of them synonymous; and 3 mutations at exon 13 (8.3%; 3/36 mutations), in 2 tumour samples, 1 synonymous, 1 missense and 1 frameshift mutation (c.14406 Ins. C).

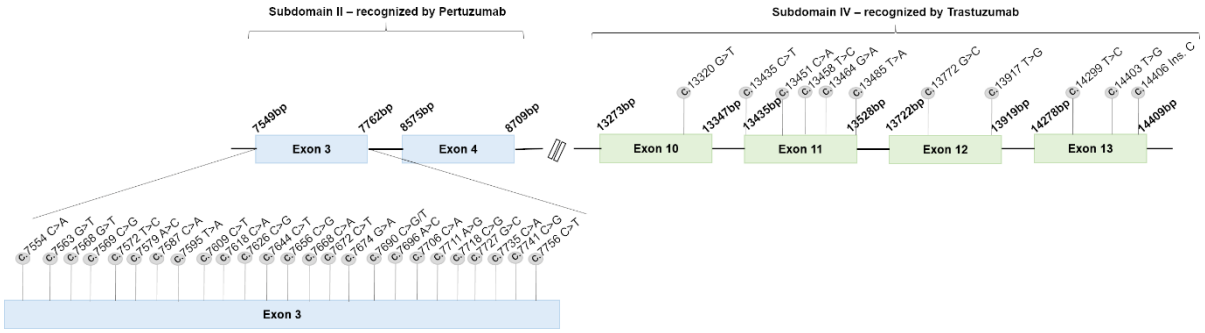

Figure 18. Mutations identified in the feline *her2* ECD, subdomains II and IV were not reported as induce resistance to therapy. All the mutations found occur in a low frequency (n= 1), being more common in exon 3, subdomain II (69.4%). Any of the described mutations were reported in breast cancer patients as induce therapeutic resistance.

Additionally, any correlation was reported between animals' clinicopathological features and the described mutations. Moreover, after comparing the feline mutations with the genomic human *her2* ECD sequence, and searching in the referred databases, the mutations found were never reported as inducing resistance to anti-HER2 immunotherapy, in breast cancer patients.

4. Discussion

The FMC is a common disease, showing similar features with human breast cancer (Maria Soares, Ribeiro, et al. 2016; Soare and Soare 2019). Presently, a lack of therapeutic options combined with a late diagnosis have a severe impact on overall survival and disease free survival of cats with mammary carcinoma. Thus, to overcome this problem, and since the cat is a good cancer model (Porrello et al. 2006), the antiproliferative effects of two anti-HER2 mAbs (trastuzumab and pertuzumab) and one ADC (T-DM1) were evaluated, using FMC cell-based models (CAT-MT, FMCm and FMCp cell lines), presenting different HER2 expression levels, and with no mutations described as conducing to resistance to therapy (**Table S2.3 – Annex II**).

Currently, only one felinized mAb is known to be in use as a therapeutic option for cats (Gearing et al. 2016; Enomoto et al. 2019), with no information about the use of mAbs in the treatment of the FMC. Furthermore, despite the reproducible (Hegde et al. 2007; Y. Zhang et al. 2016) and promising results obtained by testing human TKi, approved for breast cancer patients (lapatinib and neratinib), in FMC cell-based models, achieving 100% of cytotoxicity by the use of lapatinib (average IC_{50}= 8756 nM±83), and a maximum of 79.4% of cytotoxicity in the FMCp cell line, by exposure to neratinib (Gameiro, Almeida, et al. 2021), no clinical trials are performed. In cats, the approved TKi are not specific for the mammary tumours, and are associated to severe side effects (Bonkobara 2015). Thus, the immunotherapy protocols, using mAbs could arise has an alternative to chemotherapy. Accordingly, trastuzumab avoids HER2 homodimerization and its ECD shedding, leading to HER2 endocytic destruction. Furthermore, this mAb promotes an immune activation (Valabrega et al. 2007), inducing apoptosis, by the reduction of the anti-apoptotic Mcl-1 protein expression (Henson et al. 2006), and the inhibition of PI3K/AKT (Mohsin et al. 2005), a crucial cell survival pathway (Maniscalco et al. 2012). This study demonstrated that trastuzumab presented similar effects between the feline and human cell lines (Hofstaedter et al. 2007; Zhang et al. 2020), being possible to report a maximum of 92.6% of cytotoxicity for the CAT-MT cells and 82.7% of cytotoxicity for the FMCm cells, both HER2-positive. Particularly, for the FMCm cell line it was demonstrated that the HER2 expression levels are an upstream activator of the AKT pathway (Maniscalco et al. 2012), demonstrating this mAb an important effect, by blocking the HER2 protein and contradicting this cycle. On the other hand, for the HER2-negative FMCp cell line, the cytotoxic effect obtained was the lowest (60.1% of cytotoxicity), suggesting that the lack of HER2 receptor decrease trastuzumab efficacy (Yao et al. 2017). Additionally, such as described in other study, the HER2 expression in the FMCp cell line is not completely null (Maniscalco et al. 2012; Maniscalco et al. 2013). This result could be associated to the expression of an activated HER2 (Y877) protein, already described in triple-negative human tumours, being responsible for the benefits of trastuzumab in some breast tumour patients (Burguin et al. 2020). Furthermore, similar results between cell lines were obtained by the use of pertuzumab, as reported in other studies (Hofstaedter et al. 2007; Weigelt et al. 2010; Diermeier-Daucher et al. 2011). Pertuzumab inhibits heregulin induced heterodimerization HER2-HER3 (Franklin et al. 2004), with a decrease in the activation of the PI3K (Metzger-Filho et al. 2013) and ERK pathways (Agus et al. 2002). Analysing this assay, it was possible to obtain 60.2% and 61.8% of cytotoxicity, for CAT-MT and the metastatic FMCm cell lines, respectively. Interestingly, similarly to the trastuzumab effect, FMCp cells exposed to pertuzumab, revealed a good cytotoxic response (52.1% of cytotoxicity). In fact, pertuzumab was suggested for the treatment of triple-negative human breast cancer, which express the circular HER2 RNA, encoding for HER2-103, as putatively occur with the FMCp cell line (Maniscalco et al. 2013), which is

antagonized by pertuzumab (Li et al. 2020). Finally, the ADC T-DM1, allows a selective delivery of the DM-1 molecule to the HER2-expressing tumour cells, preventing the HER2 homodimerization, and inhibiting the microtubule assembly (Lambert and Chari 2014), which induces cell apoptosis (Barok et al. 2014; Lambert and Chari 2014; Liu et al. 2020), by blocking the AKT/mTOR pathway (Liu et al. 2020). In the experimental assay, T-DM1 was tested in lower concentrations, comparing to mAbs, and showing superior cytotoxic effects, directly dependent on the cell membrane HER2 concentration (Barok et al. 2014). In fact, 94.0% of cytotoxicity was obtained for the CAT-MT cells, presenting a HER2 2+ score, and 53.8% of cytotoxicity for the metastatic FMCm cells, with a HER2 1+ score. Interestingly, the cytotoxic effect of T-DM1 in the FMCp cell line presented promising results, being obtained 74.2% of cytotoxicity, which could be explained by the axis DM-1/cytoskeleton-associated protein 5 (CKAP5) a microtubule regulator protein, cell surface target for T-DM1, inducing cytotoxicity in no HER2-overexpressing cells (Endo et al. 2018), suggested as a target in triple-negative breast cancer therapy (Nagayama et al. 2020). Furthermore, we were unsuccessful to reproduce the cytotoxic effect in the SkBR-3 cell line after T-DM1 exposure (Barok et al. 2011; Sabbaghi et al. 2017; Endo et al. 2018), obtaining 50.5% of cytotoxicity, at the maximum concentration tested (1000 µg/mL). Despite the general characteristics of the tumour are maintained (**Figure S2.1 – Annex II** and **Table S2.1 – Annex II**), this result could be explained due to molecular changes along cell culture (Zaitseva et al. 2006; Januszyk et al. 2015). Unfortunately, it was not possible to achieve 100% of cytotoxicity, with any of the compounds tested, which could be explained due to a need of a 3D system, allowing a proper antigen-antibody conformational interaction (Weigelt et al. 2010; Tatara et al. 2018).

The tested mAbs and the ADC cell death mechanism occur by apoptosis, suggesting a Bcl-2 dependent mechanism (Hui-Wen Lo 2013), conserved between the feline and human cell lines (Henson et al. 2006; Barok et al. 2014; Yamashita-Kashima et al. 2017). Furthermore, the majority of the cells occurred in late phase apoptosis after drug exposure, presenting a permeabilized cell membrane (Poon et al. 2010) (Annexin positive *vs.* PI positive). Nevertheless, in the FMCp cell line a higher percentage of cells occur in early phase apoptosis (Annexin positive *vs.* PI negative), for all the compounds tested, suggesting the need of a more prolonged exposure time to achieve the late phase apoptosis.

As for human breast cancer, combined protocols revealing to be a valuable tool (T. Liu et al. 2011; Tsang and Finn 2012; Tóth et al. 2016) in the feline mammary tumour cell lines, presenting antiproliferative synergistic effects, by the use of smaller drug concentrations, and preventing the acquired resistance to therapy, known for trastuzumab (Yuhong Lu, Xiaolin Zi, Yunhua Zhao, Desmond Mascarenhas 2001; Nagata et al. 2004; Wehrman et al. 2006; Agarwal et al. 2009; Gagliato et al. 2016; Watanabe et al. 2019), as well as for pertuzumab (Hutcheson et al. 2007). In the conjugation trastuzumab plus pertuzumab the obtained

response was known by block heterodimers formation, cancelling the influence of HER2-overexpression on cell cycle and blocking the signalling trough AKT (Nahta et al. 2004). This combination reveals to be valuable for all the feline cell lines, with the highest increase in cytotoxicity in the FMCm cell line (from 15.6% to 45.1% of cytotoxicity). Better results were obtained, considering the conjugations of mAbs plus the TKi, lapatinib, already approved for combined protocols in human breast cancer (Scaltriti et al. 2009; Stanley et al. 2017; Z.Q. Xu et al. 2017), and revealing valuable in FMC cell-based models (Gameiro, Almeida, et al. 2021). In this combined treatments similar results to the SkBR-3 cell line (Canonici et al. 2019) were obtained, revealing all the combinations a synergistic behaviour. The best conjugation reveals to be trastuzumab plus lapatinib, with a highest increase of 71.9% of cytotoxicity (from 6.52% to 78.4% of cytotoxicity), in the CAT-MT cell line. This combination is valuable since the TKi enhances trastuzumab mediated ADCC, by upregulate the HER2 expression (Scaltriti et al. 2009). Nevertheless, the conjugation pertuzumab plus lapatinib also presented a good improvement of the antiproliferative effects, with the highest increase of 69.4% of cytotoxicity (from 7.4% to 76.8% of cytotoxicity), also occurring in the CAT-MT cell line. In this study we associated mAbs with the reversible TKi, lapatinib, but combined protocols of mAbs with the irreversible TKi, neratinib, have been revealing valuable tools for the treatment of breast cancer patients (Blackwell et al. 2019). Furthermore, considering the promising results obtained by the use of neratinib, in the FMC cell lines (Gameiro, Almeida, et al. 2021), more studies are need with neratinib in conjugation assays, to unveil different possibilities for the treatment of cats with mammary carcinomas.

Beyond the acquired therapy resistance, somatic mutations in the *her2* ECD could explain the resistance patterns obtained with the use of anti-HER2 molecules, despite mutations in the *her2* gene are described as more common in the TK domain (Gaibar et al. 2020), and in triple-negative tumour subtype (Mishra et al. 2017). In the FMC clinical samples, mutations revealed to occur with higher frequency in the luminal B subtype (61.1%). In the studied population, the majority of the *her2* ECD mutations occurred in exon 3 (69.4%), subdomain II, which encode for the region recognized by pertuzumab, suggesting that if resistance occurs in cat, it might be more common in therapeutic protocols using this mAb. Additionally, 38.9% (14/36 mutations) of the mutations were synonymous and 61.1% (22/36 mutations) corresponds to missense mutations, conditioning 59.1% (13/22 mutations) of them a change in the polarity of the codified codon, which could modify the 3D arrange of the protein (Rajasekaran et al. 2008). In breast cancer patients, one of the most described mutation in the *her2* ECD is S310F/Y, related to a resistant pattern to trastuzumab (Hyman et al. 2018) and pertuzumab (Connell and Doherty 2017; Gaibar et al. 2020) therapies, not reported in our population. Furthermore, none of the mutations found in the *her2* ECD were described as inducing resistance to therapy, according to National Cancer Institute, International Cancer

Genome Consortium and COSMIC databases, and none of them were related to the cat's clinicopathological features.

5. Conclusions

This study tested two mAbs (trastuzumab and pertuzumab) and an ADC compound (T-DM1) revealing valuable *in vitro* antiproliferative effects in feline primary (CAT-MT and FMCp) and metastatic (FMCm) cell lines, as well as a conserved cell death mechanism, by apoptosis, comparing to the human breast cancer cell line, SkBR-3. Furthermore, promising synergistic antiproliferative effects were obtained by combining both mAbs (trastuzumab plus pertuzumab), and mAbs with the TKi, lapatinib, already referred as valuable therapeutic protocols for humans. Additionally, mutations found in the clinical samples were never reported as inducing resistance to therapy, in breast cancer patients. Concluding, the use of anti-HER2 mAbs and combined protocols are proposed as a new targeted therapy, for cats with different mammary carcinoma subtypes, suggesting that mAbs-resistant FMCs are rare. Still, more studies are need to produce felinized anti-HER2 monoclonal antibodies, preventing cat's immune reactions. Moreover, the similarities between the FMC and human breast cancer were demonstrated, reinforcing the utility of the cat as a breast cancer model.

Author Contributions

Conceptualization, A.G. and F.F.; Methodology, A.G. and F.F.; Formal analysis, A.G., C.N. and FF.; Investigation, A.G., C.N., J.C. and F.F; Supervision, F.F.; Funding acquisition, F.F; Project administration, F.F.; Writing – original draft preparation, A.G. and F.F.; Writing – review and editing, A.G., C.N., J.C., and F.F. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The authors would like to thank to Raffaella De Maria, University of Turin, Department of Veterinary Sciences, Italy for the CAT-MT cell line, Nobuo Sasaki (DVM, PhD) and Takayuki Nakagawa (DVM, PhD), from Graduate School of Frontier Science, University of Tokyo, Japan, for the FMCm and FMCp cell lines, and Luís Costa, Molecular Medicine Institute, University of Lisbon, Portugal, for the human SkBR-3 cell line. Moreover, we want to thank to Fátima Cardoso, Champalimaud Foundation, Lisbon, Portugal for the mAbs and the ADC used in this study, and Filipe Almeida, National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal for the technical support on the analysis of the cytotoxicity assays.

Chapter IV

Experimental Work

Serum and tissue expression levels of leptin and leptin receptor are putative markers of specific feline mammary carcinoma subtypes

Andreia Gameiro, Catarina Nascimento, Ana Catarina Urbano, Jorge Correia and Fernando Ferreira (2021), Frontiers in Veterinary Science, DOI: 10.3389/fvets.2021.625147

Abstract

Obesity is an established risk factor for breast cancer in post-menopausal women, being associated with elevated serum levels of leptin. Although overweight is a common condition in cat, the role of leptin and its receptor in feline mammary carcinoma remains unsettled. In this study, serum leptin and leptin receptor (ObR) levels were investigated in 58 cats with mammary carcinoma and compared with those of healthy animals, as were the expression levels of leptin and ObR in tumour tissues. The results showed that the Free Leptin Index is significantly decreased in cats with mammary carcinoma (p= 0.0006), particularly in those with luminal B and HER2-positive tumours, and that these animals also present significantly lower serum leptin levels (p< 0.01 and p< 0.05, respectively). Interestingly, ulcerating tumours (p= 0.0005) and shorter disease-free survival (p= 0.0217) were associated to serum leptin levels above 4.17 pg/mL. In contrast, elevated serum ObR levels were found in all cats with mammary carcinoma (p< 0.001), with levels above 16.89 ng/mL being associated with smaller tumours (p= 0.0118), oestrogen receptor negative status (p= 0.0291) and increased serum levels of CTLA-4 (p= 0.0056), TNF-α (p= 0.0025), PD-1 (p= 0.0023) and PD-L1 (p= 0.0002). In tumour samples, leptin is overexpressed in luminal B and triple-negative carcinomas (p= 0.0046), whereas ObR is found to be overexpressed in luminal B tumours (p= 0.0425). Altogether, our results support the hypothesis that serum levels of leptin and ObR can be used as biomarkers of specific feline mammary carcinoma subtypes, and suggests the use of leptin antagonists as a therapeutic tool, reinforcing the utility of the cat as a cancer model.

Keywords: feline mammary carcinoma; leptin; leptin receptor; free leptin index; biomarkers

1. Introduction

Feline mammary carcinoma (FMC) is a high prevalence disease (12 to 40% of all tumours in cat) that shows similar clinicopathological and genetic features (Dagher et al. 2019), comparing to human breast cancer (Maria Soares, Ribeiro, et al. 2016), supporting its use in comparative oncology studies (Vail and Macewen 2000; Porrello et al. 2006), and allowing to improve therapeutic protocols for women and cats (Ferreira, Martins, et al. 2019). Despite the cat is considered a suitable cancer model, especially for the most aggressive mammary carcinomas subtypes, HER2-positve (De Maria et al. 2005; Maria Soares, Ribeiro, et al. 2016) and triple-negative (De Maria et al. 2005; Wiese et al. 2013; Caliari et al. 2014), further efforts are needed to track disease progression (Zappulli et al. 2015). Some common biomarkers are already identified, as for example, the androgen receptor (Dagher et al. 2019), the PD-1 (Nascimento et al. 2020) and the CTLA-4 (Urbano et al. 2020), which represent potential molecular therapeutic targets. Likewise, obesity is a common nutritional disorder in the cat, with higher prevalence in indoor and sterilized animals above three years of age (Shibata et al. 2003). In humans, obesity induces a chronic inflammatory status, being a risk factor for breast cancer (Hosney et al. 2017; Pan et al. 2018; Modzelewska et al. 2019).

Leptin is a 16 kDa adipocytokine, encoded by the *obese* gene and involved in the central regulation of food intake, energy homeostasis, modulation of reproductive function and peripheral metabolic processes, such as breast/mammary gland development, cellular proliferation and angiogenesis (Cao et al. 2001; Jéquier 2002; Andò and Catalano 2012). In tissues and serum, leptin expression is modulated by fat mass, with healthy cats showing lower serum leptin levels than obese animals (Shibata et al. 2003), as reported in humans (Hosney et al. 2017; Pan et al. 2018). Interestingly, although this protein is mainly secreted by adipocytes, it can also be expressed by pathologically altered cells, such as cancer cells (Artac and Altundag 2012; Bonofiglio et al. 2019). Thus, malignant cells can regulate their metabolic activities (Kang and Moon 2010), promoting uncontrolled cell growth via Wnt/β-catenin (Liang et al. 2018), migration, invasion and angiogenesis (Kim 2009; Modzelewska et al. 2019), and downregulating apoptosis through a Bcl-2-dependent mechanism (Artwohl et al. 2002; Kang and Moon 2010). Accordingly, leptin overexpression is detected in breast cancer cells and neighbouring adipocytes, contrasting with normal breast glandular epithelial cells (Khabaz et al. 2017; Modzelewska et al. 2019), promoting the expression of several tissue factors (Mahbouli et al. 2017), which suggest an oncogenic role for this adipocytokine (Hosney et al. 2017). Furthermore, studies in human breast cancer patients showed that leptin overexpression has paracrine effects, not always reflected in serum levels, but associated with more aggressive tumours and therapy resistance (Khabaz et al. 2017). Additionally, in overweight human patients a positive correlation was found between leptin overexpression in

the tumour microenvironment and oestrogen receptor (ER)-positive breast cancer, and with a human epidermal growth factor receptor 2 (HER2)-positive status frequently related to a more invasive tumour phenotype (Cha et al. 2012).

In parallel, the leptin receptor (ObR, 150-190 kDa) was found to be involved in innate and adaptive immunity (Gorska et al. 2010), being expressed in several organs, including breast and peripheral tissues, as well as in adipocytes (Appelton et al. 2000; Sánchez-Jiménez et al. 2019) and immune cells. ObR has an extracellular N-terminus domain, a transmembrane domain and a cytoplasmic C-terminus domain. Upon leptin ligation, ObR homodimerizes and the associated JAK monomer is auto-phosphorylated to activate the downstream signalling pathways (Artac and Altundag 2012); in the case of ObR forms with lack of auto-phosphorylation capabilities, auxiliary kinases are important (Sánchez-Jiménez et al. 2019). The soluble ObR form is a 146 kDa protein (Najiba Lahlou, Karine Clement, Jean-Claude Carel 2000) that can be generated by cellular apoptosis or by the proteolytic cleavage of the extracellular anchored protein domain, with this shedding being more frequent in shorter intracellular isoforms. In serum, ObR modulates the leptin bioavailability, being decreased in obese humans (Artac and Altundag 2012). In breast cancer patients, ObR is overexpressed independently of the ER status (Hosney et al. 2017), being correlated with low overall survival (OS) (Bonofiglio et al. 2019). Furthermore, the ratio between leptin/ObR serum levels (Free Leptin Index – FLI) is considered a useful predictor of leptin activity, reflecting the individual metabolic status (Owecki et al. 2010) and when increased it is an important risk factor for breast cancer development (Rodrigo et al. 2017). In parallel, studies in breast cancer patients found an association between leptin and ObR overexpression with a chronic inflammatory status, conditioning T-cell immune responses (increase Th1- and decrease Th2-responses) (Zhang et al. 2002) and the activation of immune checkpoint inhibitors (Sánchez-Jiménez et al. 2019). Indeed, some studies in humans have shown a positive correlation between overexpression of leptin and ObR with several immunomodulatory molecules (e.g. Cytotoxic T-Lymphocyte Associated Protein 4 – CTLA-4; Tumour Necrosis Factor α – TNF-α; Programmed Cell Death-1 – PD-1 and Programmed Cell Death-ligand 1 – PD-L1) (Wei et al. 2016; Dayakar et al. 2017). While CTLA-4 is a protein related to the inflammatory response that is increased in breast cancer patients, contributing to immune downregulation (Erfani et al. 2010), TNF-α is a pro-inflammatory cytokine that induces apoptosis promoted by the absence of leptin (Singh et al. 2020). Moreover, the overexpression of PD-1 in T-cells is associated with ObR overexpression in humans with distinct tumour types (Rivadeneira et al. 2019), induced through the AKT pathway activation by oestrogens (El Andaloussi and Al-Hendy 2017) and is responsible for the PD-1 mediated T-cell dysfunction (Wang et al. 2019).
As mentioned above, obesity is associated with increased leptin levels, which induces resistance to chemotherapy (Surmacz 2013; Linares et al. 2019). Therefore, the leptin/ObR

axis has been widely studied (Guo et al. 2012) as a target for an adjuvant therapy, not only in ER-positive tumour status (Surmacz 2013), but also in triple-negative tumours (Otvos et al. 2011), in which the lack of hormonal receptors reduces the therapeutic options. Nowadays, different therapeutic strategies targeting the leptin/ObR axis are being used, namely leptin antagonists, that downregulate the leptin downstream pathways (e.g. Wnt and STAT3) (Otvos et al. 2011; Guo et al. 2012; Surmacz 2013; Zabeau et al. 2014), leptin and ObR specific monoclonal antibodies or nanoparticles, that prevent leptin/ObR binding and, finally, soluble ObR molecules that enclose plasmatic leptin, regulating its availability (Zabeau et al. 2014).

To the best of our knowledge, this study is the first to evaluate the serum leptin and ObR levels, as well as tumour tissue expression of leptin and ObR in cats with mammary carcinoma. Thus, the main goals of this study were to: 1) compare the serum leptin and ObR levels of cats with mammary carcinoma stratified by molecular subtype with those of healthy animals; 2) investigate the leptin and ObR expression in tumour tissues and compare it with normal mammary tissues; 3) search for statistical associations between serum leptin/ObR levels and leptin/ObR IHC scores in tumour mammary tissues and 4) test for statistical associations between serum leptin/ObR levels and clinicopathological features, in order to evaluate the utility of leptin and ObR as diagnostic and/or prognosis biomarkers or promising drug targets in cats with mammary carcinoma.

2. Materials and Methods

2.1. Animal population

Paired tumour and serum samples were collected from 58 female cats, with fully documented history of FMC, exhibiting a mean age at diagnosis of 11.5 years (range 6.5-18 years), with the majority showing an undifferentiated breed and presenting an average body condition score (1-9) of 3.73 (ranging between 1 and 7). Also, 24 serum samples from healthy cats presented for elective ovariohysterectomy showing a mean age of 1.37 years (range 0.5-5.5 years) and an average body condition score (1-9) of 5.0 (ranging between 4 and 6), were collected at the Teaching Hospital of the Faculty of Veterinary Medicine, University of Lisbon. All the procedures involving manipulation of animals were consented by the owners. For each animal enrolled in the study, the clinicopathological data were recorded, including: age; breed; body weight; reproductive and contraceptive administration status; treatment status (none, mastectomy or mastectomy plus chemotherapy); number, location, size, and histopathological classification; ER status, PR status, HER2 status (Maria Soares, Madeira, et al. 2016) and Ki-67 index (M. Soares, Ribeiro, Carvalho, et al. 2016) of tumour lesions; malignancy grade, scored using the Elston & Ellis system (Elston, C.W. and Ellis 1998); presence of tumour necrosis, lymphatic invasion, lymphocytic infiltration, and/or cutaneous ulceration; regional lymph node involvement; and clinical stage (TNM system; **Table 11**). Regarding the molecular

subtyping of feline mammary carcinomas (Goldhirsch et al. 2013; Maria Soares, Ribeiro, et al. 2016), animals were stratified in luminal A (n= 10), luminal B (n= 17), HER2-positive (n= 15) and triple-negative (n= 16) groups. The animals were anesthetized before surgical procedures and blood samples were collected without interfering with the animals' well-being. Briefly, all tissue samples were embedded in paraffin after fixation in 10% buffered neutralized formalin (pH 7.2), during 24-48 hours, while serum samples were separated from clotted blood by centrifugation (1500 g, 10 minutes, 4ºC) and stored at -80ºC until further use. All samples that showed haemolysis were discarded, as recommended (Lippi et al. 2012; Maria Soares, Ribeiro, et al. 2016).

Table 11. Clinicopathological features of the female cats with mammary carcinomas enrolled in this study. (n=58)

Clinicopathological feature	Number of animals (%)	Clinicopathological feature	Number of animals (%)
Breed		**Size**	
Undifferentiated	44 (75.9%)	<2cm	22 (37.9%)
Siamese	7 (12.1%)	≥2cm	36 (62.1%)
Persian	5 (8.6%)	**Animal Weight**; 23 unknown	
Norwegian Forest	2 (3.4%)	<3kg	6 (10.3%)
Age		3 – 5kg	24 (41.4%)
<8years old	4 (6.9%)	>5kg	5 (8.6%)
≥8 years old	54 (93.1%)	**Treatment**; 3 unknown	
Reproductive status; 1 unknown		Mastectomy	49 (84.5%)
Spayed	20 (34.5%)	Mastectomy + Chemo	4 (6.9%)
Pill	21 (36.2%)	None	2 (3.4%)
Both	9 (15.5%)	**Multiple tumours**	
Any	7 (12.1%)	Yes	35 (60.3%)
Lymph node status; 4 unknown		No	23 (39.7%)
Positive	19 (32.8%)	**Malignancy grade**; 1 unknown	
Negative	35 (60.3%)	I	3 (5.2%)
Stage (TNM)		II	8 (13.8%)
I	15 (25.9%)	III	46 (79.3%)
II	6 (10.3%)	**Necrosis**	
III	31 (53.4%)	Yes	42 (72.4%)
IV	6 (10.3%)	No	16 (27.6%)
Lymphatic invasion		**Lymphocytic infiltration**; 2 unknown	
Yes	7 (12.1%)	Yes	37 (63.8%)
No	51 (87.9%)	No	19 (32.8%)
HER2 status		**Tumour ulceration**	
Positive	14 (24.1%)	Yes	8 (13.8%)
Negative	44 (75.9%)	No	50 (86.2%)
ER status		**Ki67 index**; 1 unknown	

Positive	31 (53.4%)	Low (<14%)	18 (31%)
Negative	27 (46.6%)	High (≥14%)	39 (67.2%)
PR status			
Positive	36 (62.1%)		
Negative	22 (37.9%)		

TNM – Tumour, Node, Metastasis; ER – Oestrogen receptor; PR – Progesterone receptor

2.2. Measurement of serum leptin, ObR, CTLA-4, TNF-α, PD-1 and PD-L1 levels

The serum levels of leptin and ObR, CTLA-4, TNF-α (Urbano et al. 2020), PD-1 and PD-L1 (Nascimento et al. 2020) were quantified by using commercial ELISA-based kits (R&D Systems, Minneapolis, USA; DY398-05, DY389, DY476, DY2586, DY1086 and DY156, respectively). For each assay, a standard curve was plotted using 10-fold serial dilutions of the recombinant proteins provided by the manufacturer, and the r^2 values were calculated using a quadratic regression [r^2= 0.9976 for leptin, r^2= 0.9632 for ObR, r^2=0.99 for PD-1 and r^2=0.96 for PD-L1 (Nascimento et al. 2020)], whereas serum CTLA-4 and TNF-α concentrations were determined by using a curve-fitting equation (r^2> 0.99), as previously reported (Urbano et al. 2020). Briefly, a 96-well plate was prepared by adding the capture antibody to each well and incubate overnight. Plates were then treated with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 hour, to prevent nonspecific binding. Standards and diluted serum samples were added to sample wells and incubated for 2 hours at room temperature (RT), followed by incubation with the detection antibody for 2 hours at RT. Afterwards, the streptavidin-conjugated to horseradish peroxidase (HRP) was added to each well and incubated at RT for 20 minutes previous to the addition of the substrate solution in 1:1 H_2O_2 and tetramethyl-benzidine to each well (20 minutes at RT in the dark). The reaction was interrupted by adding a stop solution ($2NH_2SO_4$) and the absorbance was measured by a spectrophotometer (FLUOStar OPTIMA, Microplate Reader, BMG, Ortenberg, Germany), using 450 nm as the primary wavelength and 570 nm as a reference wavelength. After serum leptin and ObR measurement, the FLI was calculated based on the ratio between leptin/ObR serum levels (Owecki et al. 2010).

2.3. Assessment of the leptin and ObR status by immunohistochemistry (IHC)

Initially, the feline mammary carcinoma formalin fixed paraffin-embedded (FFPE) samples were stained with haematoxylin-eosin to select a representative tumour area (n= 58) and a normal tissue area to be used as control (n= 20). FFPE samples were sectioned in slices with 3 µm thickness (Microtome Leica RM135, Newcastle, UK) and mounted on a glass slide (SuperFrost Plus, Thermo Fisher Scientific, Massachusetts, USA). On PT-Link module (DAKO, Agilent, Santa Clara, USA), samples were deparaffinised, hydrated and antigen retrieval was performed for 20 minutes at 96ºC using Tris-EDTA buffer pH 9.0 (EnVision™ Flex Target Retrieval Solution High pH, DAKO). Then, slides were cooled for 30 minutes at RT and

immersed twice for 5 minutes in distilled water. IHC technique was performed with commercial solutions from the Novolink™ Max Polymer Detection System Kit (Leica Biosystems, Newcastle UK). Before antibody incubation, tissue samples were treated with Peroxidase Block Novocastra Solution (Leica Biosystems) for 15 minutes and the unspecific antigenic recognition was inhibited by incubation with Protein Block Novocastra Solution (Leica Biosystems) for 10 minutes. Finally, tissue samples were incubated at RT for 1 hour, in a humidified chamber, with the following primary antibodies: anti-leptin antibody (ab3583, Abcam, Cambridge, UK) and anti-ObR antibody (ab104403, Abcam), both diluted at 1:200. The slides were washed twice, for 5 minutes, between all the incubation steps, using a PBS solution at pH 7.4. Then, the detection polymer was incubated for 30 minutes at RT, and detection was performed using diaminobenzidine (DAB substrate buffer and DAB Chromogen, Leica Biosystems) for 5 minutes. Later, samples were counterstained with Gills haematoxylin (Merck, New Jersey, USA) for 5 minutes, dehydrated in an ethanol gradient and xylene, and mounted using Entellan mounting medium (Merck). Antibodies were predicted to react with the feline proteins, occurring in cell membrane and cytoplasm (Ishikawa et al. 2004; Dall'Aglio et al. 2012). Human breast tissue and feline liver were used as positive controls for the leptin staining being expressed in the glandular cells (Ishikawa et al. 2004; Khabaz et al. 2017), and hepatocytes (Otte et al. 2004; Perumal et al. 2020), presenting a faint staining in cholangiocytes (Fava et al. 2008), respectively. For the ObR, human and feline kidney were used as positive controls, showing a immunostaining in tubular and some glomerular cells (Hardwick et al. 2001; Perumal et al. 2020), in both species, as reported. Sections of the feline mammary tissues analysed were used as a negative controls.

Leptin and ObR were evaluated in the glandular epithelium of the tumour, stromal tissue and tumour infiltrating inflammatory cells. To access proteins immunoreactivity we used a previously reported scoring system (Ishikawa et al. 2004; Hosney et al. 2017; Khabaz et al. 2017) and the H-Score published by the American Society of Clinical Oncology (ASCO). The final IHC score was obtained by multiplying the positive cells (0= absence of staining; 1= all cells stained), by the highest staining intensity (**Table 12**), varying from 0 to 3, with tissue samples scored as 0 considered negative, and samples scored as 3 as highly reactive. All slides were subjected to blind scoring, by two independent and experienced pathologists.

Table 12. Scoring criteria of immunostaining assay for leptin and ObR. Three microscopic fields were analysed at 400x magnification.

	Staining intensity	
Stained tumour cells	**Score**	**Interpretation**
(0-1)	0	No staining
	1	Weak

	2	Moderate
	3	Strong†

Final IHC score = stained tumour cells x staining intensity score (0 – 3)

†High protein reactivity

2.4. Statistical analysis

Statistical analysis was carried out using the GraphPad Prism software, version 5.04 (California, USA), with two-tailed *p*-values less than 0.05 considered statistically significant for a 95% confidence level (**p*< 0.05, ***p*< 0.01 and ****p*< 0.001) and the average values were represented with the standard deviation.

The non-parametric Kruskal-Wallis test was performed to compare leptin and ObR results between healthy cats and cats with mammary carcinomas stratified by tumour subtype. Receiver-operating characteristic (ROC) curves were performed to choose the optimal cut-off value for serum leptin and ObR levels, and to determine the specificity and sensitivity of the technique to diagnose the disease. The non-parametric Mann-Whitney test was used to compare the serum levels of both proteins with several clinicopathological features. Survival analysis was performed using the Kaplan-Meier test to evaluate the disease-free survival (DFS) in cats with mammary carcinomas. Correlations between serum ObR levels and the previously reported serum CTLA-4, TNF-α, PD-1 and PD-L1 concentrations (Nascimento et al. 2020; Urbano et al. 2020) were investigated using the Spearman's rank correlation coefficient.

3. Results

3.1. Cats with mammary carcinoma showed lower Free Leptin Index

The Free Leptin Index (FLI) was determined in the serum samples of cats with mammary carcinomas and compared with healthy animals. Results obtained showed that cats with disease had a significantly lower FLI than the control group (0.44 *vs.* 0.86, *p*= 0.0006, **Figure 19**). Moreover the same results were obtained if the outliers were removed from the analysis (*p*= 0.0005).

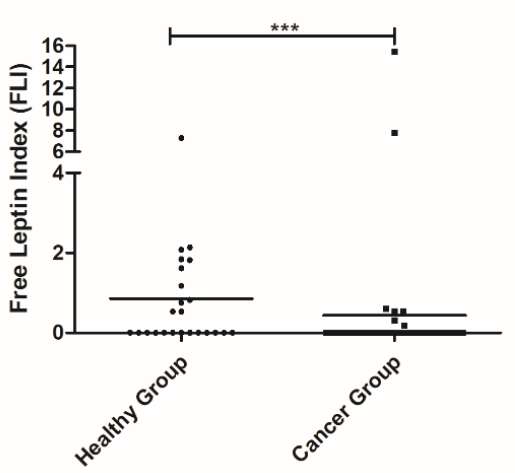

Figure 19. Dot plot diagram showing that the Free Leptin Index (FLI) was significantly elevated in healthy animals than in cats with mammary carcinoma (*p*= 0.0006).

In addition, results revealed that body weight did not influence serum leptin and ObR levels, both in the control group (*p*= 0.0760 and *p*= 0.8432, respectively, **Figure 20A** and **B**) and in the cancer group (*p*= 0.3294 and *p*= 0.9722, respectively, **Figure 20C** and **D**).

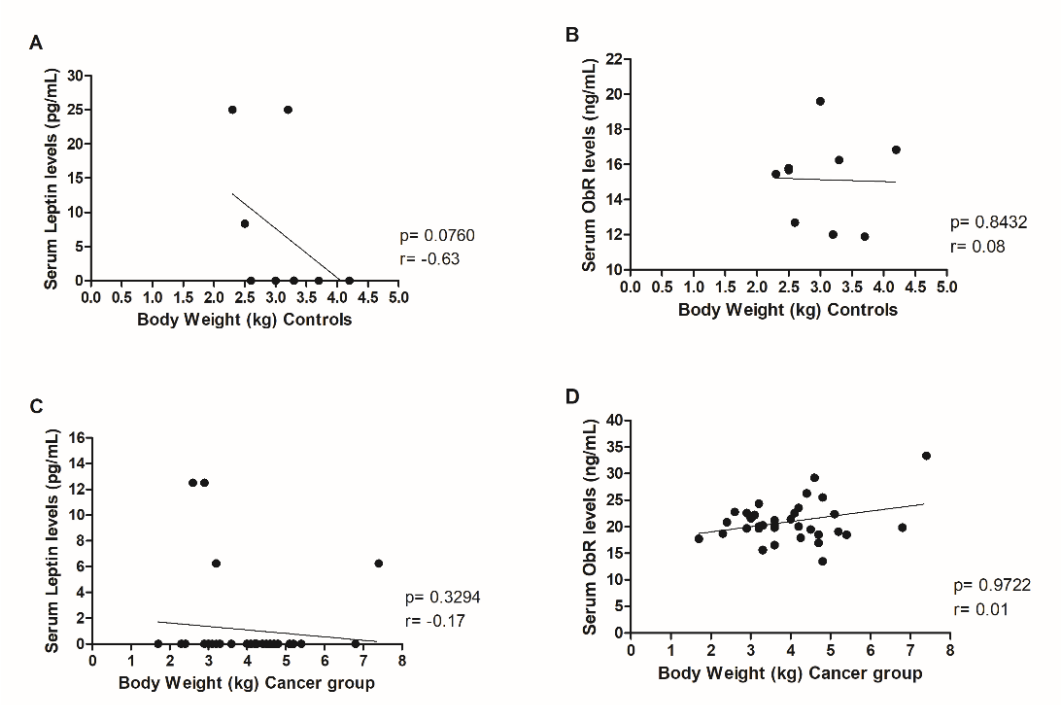

Figure 20. Body weight did not influence leptin, neither ObR serum levels in healthy and diseased animals. Correlations were not found between **A)** serum leptin (*p*= 0.0760) or **B)** ObR (*p*= 0.8432) levels and body weight in the control group. Furthermore, evaluating the cancer group, no correlations were detected between **C)** serum leptin (*p*= 0.3294) or **D)** ObR (*p*= 0.9722) levels and feline body weight.

3.2. Cats with luminal B or HER2-positive mammary carcinoma showed decreased serum leptin levels

Regarding the serum leptin levels, results obtained showed that cats with luminal B or HER2-positive mammary carcinomas had lower serum leptin levels than healthy animals (0.00

pg/mL *vs.* 13.89 pg/ml, *p*< 0.01; 0.83 pg/mL *vs.* 13.89 pg/mL, *p*< 0.05, respectively, **Figure 21A**), and considering the analysis with no outliers, the same results could be reported (*p*= 0.0021). The optimal cut-off value to predict mammary carcinoma was 4.17 pg/ml with an area under the ROC curve (AUC) of 0.7045±0.0757 (95% CI: 0.5561-0.8528, *p*= 0.0103; sensitivity= 96.9%; specificity= 43.5%; **Figure 21B**). Considering this analysis with no outliers, an AUC of 0.6732±0.0731 (95% CI: 0.5299-0.8164, *p*= 0.0158; sensitivity= 92.9%; specificity= 43.4%) was obtained, leading to the same results. Further statistical analysis, revealed that serum leptin levels above the cut-off value were associated with tumour ulceration (*p*= 0.0005, **Figure 21C**; or *p*= 0.0009, if no outliers were considered) and shorter DFS (117 *vs.* 314 days, *p*= 0.0217, **Figure 21D**; or *p*= 0.0245, if the outliers were removed).

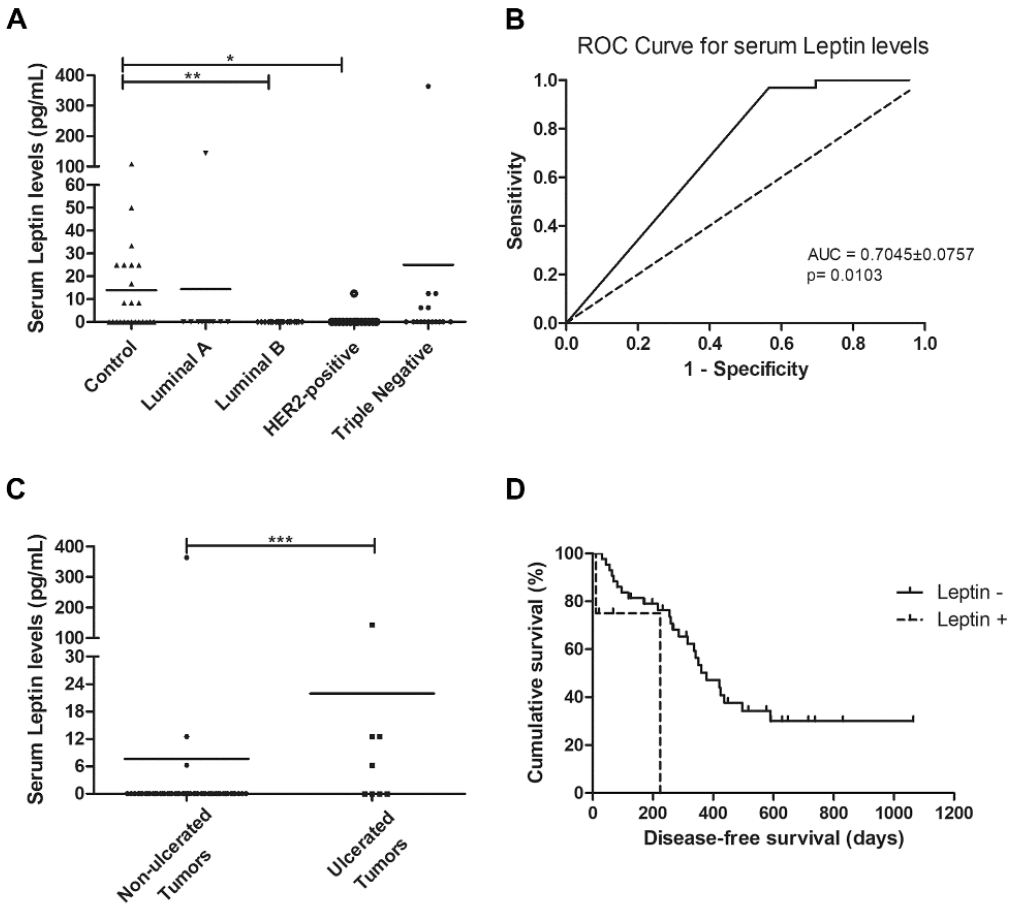

Figure 21. Cats with luminal B and HER2-positive mammary carcinomas showed decreased serum leptin levels, although cats with ulcerated tumours exhibited serum leptin levels above the cut-off value of 4.17 pg/mL, being associated with shorter disease-free survival. **A)** Dot plot diagram showing the distribution of serum leptin levels (pg/mL) among healthy animals (control) and cats stratified by the mammary carcinoma subtype. Significant decreased serum levels of leptin were found in cats presenting luminal B or HER2-positive subtypes in comparison to healthy animals (*p*= 0.0025). **B)** The optimal cut-off of serum leptin levels to predict mammary carcinoma was determined to maximize the sum of the sensitivity and specificity (4.17 pg/mL; AUC= 0.7045±0.0757, 95% CI: 0.5561-0.8528, *p*= 0.0103; sensitivity= 96.9%; specificity= 43.5%). **C)** Dot plot diagram showing that serum leptin levels were significantly higher in cats with ulcerated tumours (*p*= 0.0005). **D)** Cats with mammary carcinoma and serum leptin levels higher than 4.17pg/mL had a lower DFS (*p*= 0.0217). **p*< 0.05; ***p*< 0.01; ****p*< 0.001.

3.3. Cats with mammary carcinoma showed elevated serum levels of ObR and of inflammation mediators

Considering the above results, the serum ObR levels were also evaluated. When the animals were grouped according to the tumour subtype, a significant difference was found between the mean ranks of at least one pair of groups ($p< 0.001$, with or without outliers). Results revealed that serum ObR levels were significantly higher in animals with mammary carcinoma than in controls, independently of molecular subtype (control group 15.67 ng/ml; luminal A 23.04 ng/ml, $p< 0.001$; luminal B 20.18 ng/ml, $p< 0.001$; HER2-positive 28.99 ng/ml, $p< 0.001$; triple-negative 21.70 ng/ml, $p< 0.001$; **Figure 22A**). Furthermore, the optimal cut-off value calculated for cats with mammary carcinoma was 16.89 ng/ml, with an AUC of 0.9408±0.0288 (95% CI: 0.8842-0.9973, $p< 0.001$; sensitivity= 94.8%; specificity= 87.0%; **Figure 22B**). If the outliers were removed from the analysis, the same results were obtained, with an AUC of 0.9397±0.0293 (95% CI: 0.8823-0.9972, $p< 0.001$; sensitivity= 94.7%; specificity= 87.0%).

In addition, elevated serum ObR levels were associated with smaller tumours ($p= 0.0118$, **Figure 22C**; or $p= 0.0248$, if no outliers were considered) and with cats had an ER-negative status ($p= 0.0291$, **Figure 22D**; or $p= 0.0452$, if no outliers were considered).

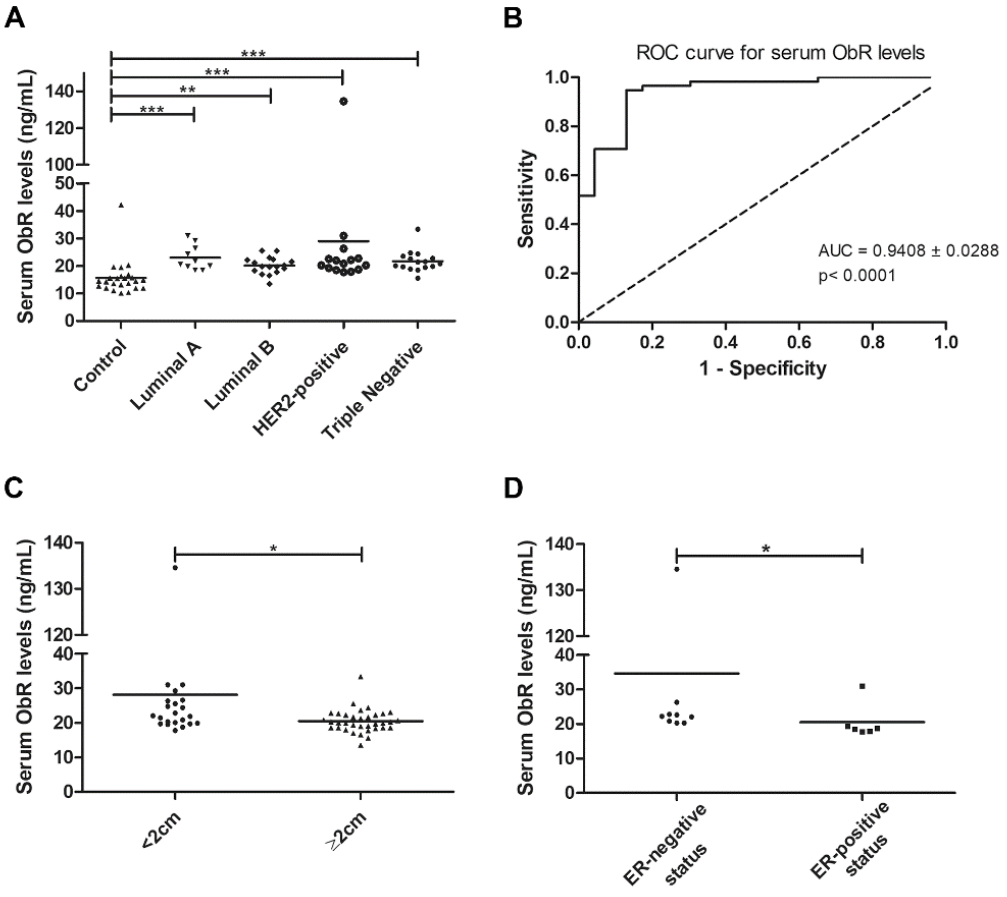

Figure 22. Cats with mammary carcinoma showed elevated serum ObR levels, with serum concentrations above 16.89 ng/mL being associated with smaller tumours and an ER-negative status.

A) Dot plot diagram showing the distribution of serum ObR levels (ng/mL) in heathy animals (control) and in cats with mammary carcinoma stratified by the molecular subtype. Significant higher serum levels of ObR were found in all tumour subtypes in comparison to healthy animals (p< 0.001). **B)** The optimal cut-off value of serum ObR levels to predict cats with mammary carcinoma was 16.89 ng/mL with an AUC of 0.9408±0.0288 (95% CI: 0.8842-0.9973, p< 0.001; sensitivity= 94.8%; specificity= 87.0%). **C)** Dot plot diagram showing that serum ObR concentrations were significantly low in tumours larger than 2 cm (p= 0.0118). **D)** Dot plot diagram displaying a positive association between higher serum ObR levels and ER-negative status (p= 0.0291). *p< 0.05; **p< 0.01; ***p< 0.001.

Finally, a positive correlation was found between serum ObR levels and serum levels of CTLA-4 (r= 0.38, p= 0.0056, **Figure 23A**), TNF-α (r= 0.40, p= 0.0025, **Figure 23B**), PD-1 (r= 0.42, p= 0.0023, **Figure 23C**) and PD-L1 (r= 0.50, p= 0.0002, **Figure 23D**). Removing the outliers from our data, the same results could be reported (CTLA-4: r= 0.34, p= 0.0153; TNF-α; r=0.37, p= 0.0064; PD-1: r= 0.39, p= 0.0002; and PD-L1: r= 0.47, p= 0.0007).

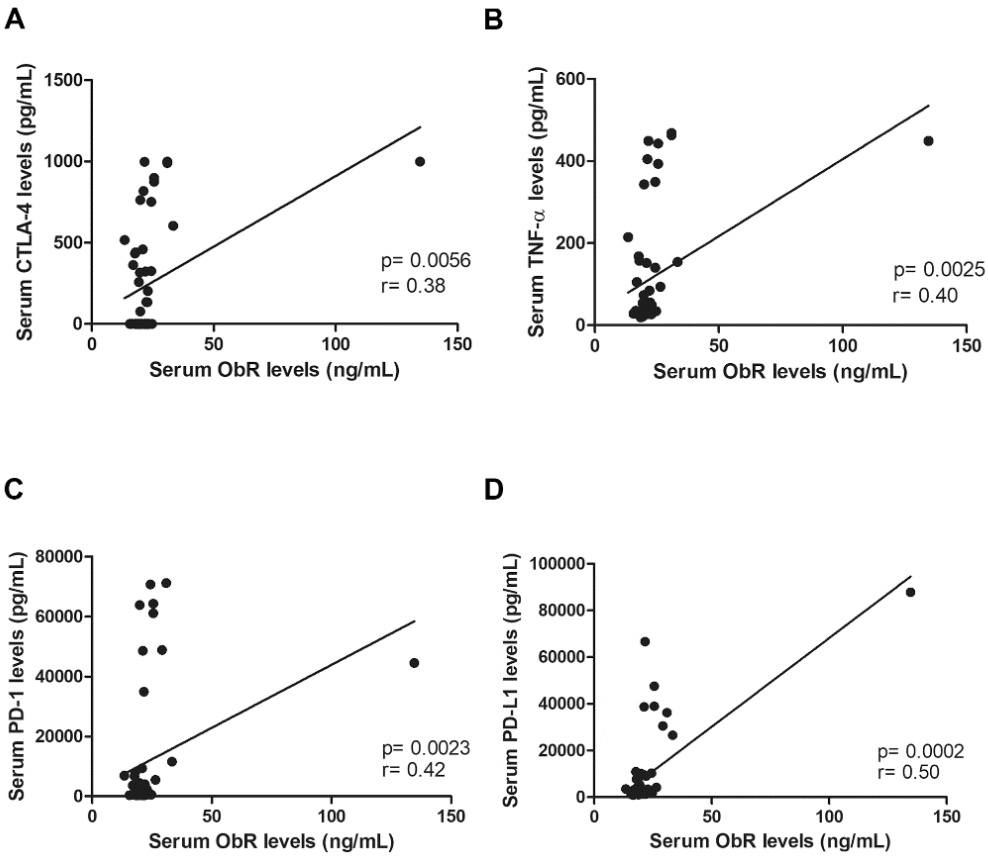

Figure 23. Serum ObR levels showed a positive correlation with inflammatory mediators, namely **A)** serum CTLA-4 levels (p= 0.0056), **B)** serum TNF-α levels (p= 0.0025), **C)** serum PD-1 levels (p= 0.0023) and **D)** serum PD-L1 levels (p= 0.0002).

3.4. Leptin and ObR are overexpressed in luminal B and triple-negative mammary carcinomas

The obtained results revealed that cats with luminal B or triple-negative mammary carcinoma showed a higher leptin IHC score in the tumour glandular cells, comparing to the healthy control samples (1.93 *vs.* 1.34, p< 0.05; 2.00 *vs.* 1.34, p< 0.05, respectively; **Figure**

24A, **Figure 25A** and **Figure 25B**). Regarding the leptin receptor, the IHC score was also significantly higher in animals with a luminal B tumour subtype than in healthy animals (2.50 *vs.* 1.75; *p*= 0.0425; **Figure 24B**, **Figure 25C** and **Figure 25D**).

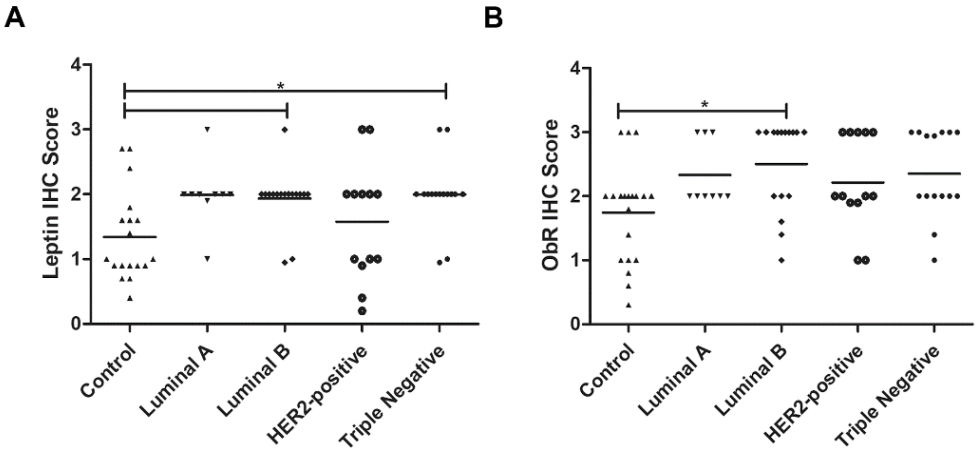

Figure 24. Final IHC scores for leptin **(A)** and ObR **(B)** in cats with mammary carcinoma stratified by the tumour subtype and compared with controls. **A)** Leptin expression was significantly higher in luminal B and triple-negative subtypes (*p*= 0.0046). **B)** Expression of ObR was statistically higher in luminal B tumour subtype (*p*= 0.0425).

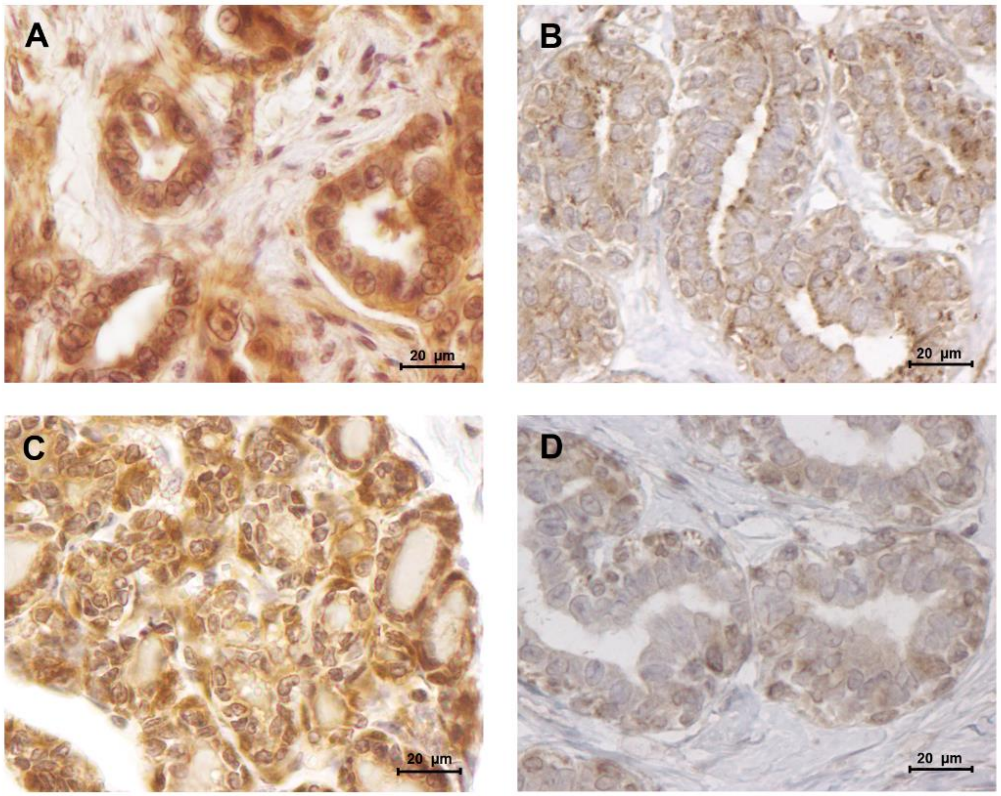

Figure 25. Leptin and ObR were overexpressed in luminal B mammary carcinomas. **A)** Leptin overexpression in a luminal B mammary carcinoma (IHC score of 1.93) contrasting with **B)** a low staining intensity detected in normal mammary tissues (IHC score of 1.34). **C)** Luminal B mammary tumours showed a higher staining intensity for ObR (IHC score of 2.50), **D)** than normal mammary tissues (IHC score of 1.75; 400x magnification).

Furthermore, the immunostaining reveals to be positive in the stroma cells, in 72.2% and 18.2% of the tumours, for leptin (IHC score of 0.79) and ObR (IHC score of 1.1), respectively. Moreover, was observed that, independently of the tumour subtype, a mean of 81% ± 2.5 of the tumour inflammatory mononuclear cells presented to be positive for the leptin staining (average IHC score of 2.0 for the macrophages and average IHC score of 1.6 for the lymphoid cells, **Figure 26A**). The same analysis, considering the ObR revealed a positive staining for a mean of 87.6% ± 2.5 of the tumour inflammatory mononuclear cells (average IHC score of 2.62 for the macrophages and average IHC score of 1.33 for the lymphoid cells, **Figure 26B**).

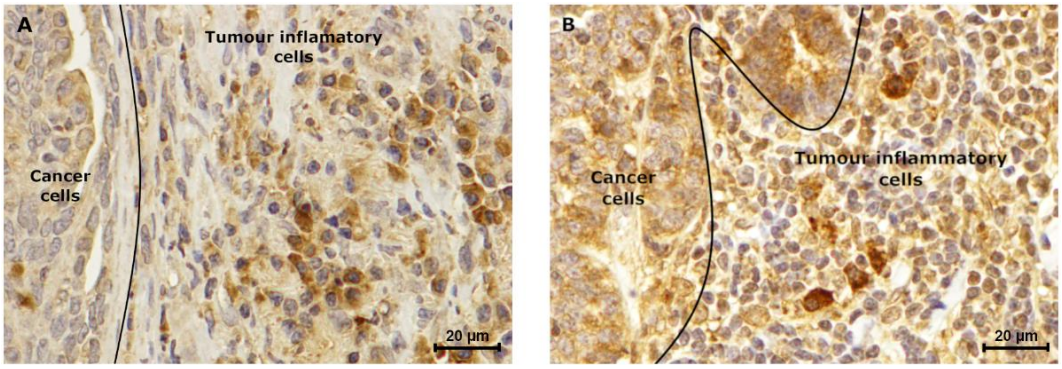

Figure 26. Tumour inflammatory cells express leptin and ObR. Luminal B carcinoma subtype showed a **A)** positive leptin staining (IHC score of 1.5), which is lower when compared to the **B)** ObR immunostaining (IHC score of 2.5) of tumour inflammatory cells. Furthermore in both samples higher staining intensity was observed in macrophages, when compared to lymphoid cells (IHC score of 2.0 *vs.* 1.2, respectively for leptin, and IHC score of 3.0 *vs.* 2.0, respectively for ObR; 400x magnification).

In addition, our findings revealed that serum ObR levels are negatively correlated with the ObR IHC score, with cats presenting higher serum ObR levels showing mammary tumours with lower ObR IHC scores (p= 0.0103, **Figure 27**; or p= 0.0244 if the outliers were removed from the analysis).

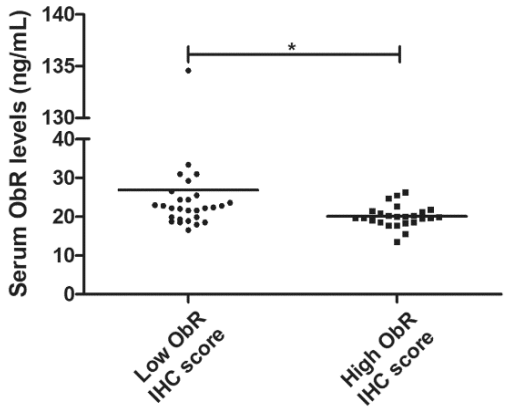

Figure 27. Dot plot diagram showing a negative correlation between serum ObR levels and tumour ObR IHC score (p= 0.0103).

4. Discussion

Although spontaneous FMC has been proposed as a suitable model for human breast cancer studies, the role of the leptin/ObR axis has never been evaluated in cats. In humans, previous studies showed that leptin and ObR overexpression are associated with pro-inflammatory and pro-tumorigenic effects, particularly in overweight women (Pan et al. 2018; Modzelewska et al. 2019). Moreover some studies reported increased serum leptin levels with aging (Li et al. 1997; Gabriely et al. 2002). In this study, the healthy group presented a mean age lower than the tumour group, and despite what is reported in human and rats (Li et al. 1997; Gabriely et al. 2002), the results obtained demonstrated that cats with mammary carcinoma have a reduced FLI in comparison to the healthy group (p= 0.0006), not only due to the increase in serum ObR levels (Chan et al. 2002), but also suggesting that diseased animals may have decreased soluble leptin levels, as reported in pre-menopausal women with breast cancer (Georgiou et al. 2016) and colon cancer patients (Arpaci et al. 2002). These results indicate that serum leptin may be recruited by mammary cancer cells to promote tumour growth and cell migration (Kim 2009). Indeed, cats with luminal B or HER2-positive mammary carcinoma showed significantly lower serum leptin levels when compared with controls (p< 0.001 and p< 0.05, respectively), revealing that serum leptin levels are downregulated in tumours with PR-positive status (Hosney et al. 2017) and/or HER2-positive status (Cha et al. 2012). In contrast, cats with luminal A showed elevated serum leptin levels, indicating that ER overexpression in the tumour may promotes leptin expression (Hosney et al. 2017). Regarding the elevated serum leptin levels found in cats with triple-negative mammary carcinomas, studies demonstrated that leptin induces cell proliferative capacity (e.g. via Wnt/β-catenin pathway) (Liang et al. 2018; Linares et al. 2019) and promotes cell survival by interacting with Bcl-2 proteins, being associated with more aggressive tumours (Crean-Tate and Reizes 2018). Indeed, our results revealed that elevated serum leptin levels occur in an advanced stage of the disease, being significantly associated with tumour ulceration (p= 0.0005) and shorter DFS (p= 0.0217), as reported for women with breast cancer (Hosney et al. 2017; Modzelewska et al. 2019).

In parallel, as documented in breast cancer patients (Garofalo et al. 2006; Hosney et al. 2017), all cats with mammary carcinoma showed higher serum ObR levels than healthy controls (p< 0.001). Also higher serum ObR levels were correlated with smaller tumour sizes (p= 0.0118), suggesting that ObR shedding occurs in small tumours, modulating the serum levels of free leptin (Gorska et al. 2010). Moreover, our results further support the hypothesis that malignant cells in larger tumours maintain the ObR expression on its surface to increase their survival and growth (Artac and Altundag 2012). Interestingly, the higher serum ObR levels were found in cats with mammary carcinomas presenting a HER2-positive/ER-negative status

(p= 0.0291), as reported for human breast cancer patients (Hosney et al. 2017), confirming the crosstalk between the leptin/ObR axis and the EGFR downstream signalling pathway (Wauman et al. 2017).

In addition, this study discloses the utility of leptin and ObR as promising diagnostic biomarkers to differentiate animals with FMC from healthy cats (cut-off value of 4.17 pg/mL for leptin and 16.89 ng/mL for ObR).

We also found that serum ObR levels were positively correlated with serum CTLA-4 (p= 0.0056), TNF-α (p= 0.0025), PD-1 (p= 0.0023) levels as reported in breast cancer patients (Rivadeneira et al. 2019), and with serum PD-L1 levels (p= 0.0002). Indeed, previous studies showed that activation of the leptin/ObR axis can result in a chronic inflammatory status (Paz-Filho et al. 2012; Wei et al. 2016), a well-known risk factor for breast cancer, with leptin being involved in CD4+ T-regulatory cells differentiation due to ObR overexpression on lymphocyte plasm membrane (Kim et al. 2010). These activated CD4+ T-regulatory cells express CTLA-4 (Wei et al. 2016) and PD-1, two immune-inhibitory checkpoint molecules that downregulate T-cell immune responses (Erfani et al. 2010), leading to tumour development (Chen 2005) and contributing to cell growth (Wang et al. 2016). On the other hand, in an attempt to control the tumorigenesis process, CD4+ T-regulatory cells secrete TNF-α (Singh et al. 2020), a molecule that shows a dual role in immunomodulation, being also expressed by cancer cells (Montesano et al. 2005), acting as an autocrine growth factor (Gautam Sethi, Bokyung Sung 2008). Altogether, these findings provide support for the crosstalk between the leptin/ObR axis and tumour immunoediting mechanisms, contributing to an immunosuppressive status in cats with mammary carcinoma (Nascimento et al. 2020; Urbano et al. 2020).

The immunostaining analysis of the tumour and normal tissue samples revealed that luminal B and triple-negative mammary carcinoma subtypes showed leptin overexpression (p< 0.05). Although a strong ObR expression was only detected in luminal B mammary carcinomas (p= 0.0425), as described in human breast cancer (Khabaz et al. 2017). Furthermore, several studies suggest that leptin and ObR are overexpressed in tumour tissues, due to hypoxia and/or as a response to insulin, IgF-1 and/or to estradiol (Garofalo et al. 2006; Cascio et al. 2008). In addition, the higher IHC scores for leptin found in luminal B carcinomas also support the previously reported association between the expression of this adipocytokine and aromatase expression, an enzyme that catalyses the conversion of androgen into oestrogen to promote tumour development via an ER-dependent mechanism (Hosney et al. 2017). The overexpression of leptin detected in triple-negative mammary carcinomas is also in concordance with previous results in triple-negative breast cancer, where leptin signalling is crucial for tumour growth (Crean-Tate and Reizes 2018; Sánchez-Jiménez et al. 2019), being associated with ERK and AKT pathways, both involved in breast cancer cells proliferation (Kim 2009). Furthermore the tumour inflammatory mononuclear cells revealed to be positive for

leptin and ObR immunostaining, with a higher proteins expression in macrophages. In fact, leptin/ObR axis are reported as activating the inflammatory response (Sánchez-Margalet et al. 2010; Paz-Filho et al. 2012). Finally, our results demonstrated that cats with low ObR-expressing mammary tumours had higher serum ObR levels, indicating a negative feedback between tumour microenvironment and serum, probably due to a shedding mechanism that leads to a reduction of serum leptin levels (Chan et al. 2002; Kim 2009). Furthermore, the data obtained emphasizes the possibility of blocking the leptin/leptin receptor axis, as an adjuvant therapy in cats with luminal B and triple-negative mammary carcinoma subtypes, as reported for breast cancer patients (Otvos et al. 2011; Guo et al. 2012; Surmacz 2013; Zabeau et al. 2014).

In conclusion, our data provide a rationale use for leptin/ObR as diagnostic and prognostic biomarkers. Indeed, cats with mammary carcinoma showed a decreased FLI, coupled with decreased serum leptin levels in animals with luminal B or triple-negative mammary carcinoma subtypes. A significant increase in serum ObR levels, was detected in all samples, independently of the tumour subtype, being associated to an immunosuppressive status. Altogether, our data indicate that cats presenting luminal B and triple-negative tumours could benefit from adjuvant therapies targeting leptin, and support the utility of spontaneous FMC as a model for comparative oncology.

Author Contributions

A.G. and F.F. designed the research; A.G., C.N., A.C.U., J.C and F.F performed the research; A.G., C. N. and F.F. analysed the data; A.G. C.N., A.C.U. and F.F. wrote the paper. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The authors would like to thank Dra. Maria Soares for the clinical samples and database.

Chapter V

Discussion, future perspectives and general conclusions

1. Discussion

The spontaneous FMC is proposed as a suitable model for HBC (Vail and Macewen 2000; Porrello et al. 2006; Soare and Soare 2019), overcoming the drawbacks of laboratory models, tested in a base of induced tumours and with poor immune responses. Although, further studies are crucial to unveil the feline mammary tumour pathogenesis and diagnosis/prognosis biomarkers, as well as, new molecular targets are need for a successful therapy. The FMC is usually diagnosed belatedly having a poor prognosis, with the HER2-positive and triple-negative subtypes, being the more common and aggressive tumours (Vu and Claret 2012; Maria Soares, Ribeiro, et al. 2016). Thus, an early detection and an effective therapy becomes crucial to improve cats' survival time.

In cats, the HER2 protein was revealed useful, as in HBC, not only as a diagnostic/prognostic biomarker, but also as a gold therapeutic target. In women breast cancer patients, several alternatives could be considered when the therapeutic protocols are designed, with the purpose of having an individual therapy, maximizing the OS and DFS times. However, in cats, therapeutic protocols for the mammary carcinoma are scarce, being most of the times only considered the mastectomy, resulting in a very poor prognosis. Thus, is urgent to develop different options for the treatment of these tumours, which could be investigated taking into account the drugs and molecular targets that present successful results in human patients. As referred, the HER2 is a common molecular target, nowadays considered not only for the HER2-positive tumours, but also in other tumour subtypes, with several drug options. In order to understand the FMC cells' response by the use of chemotherapeutic drugs, approved for HBC treatment and targeting different EGFR family members, namely TKi (lapatinib and neratinib), several *in vitro* assays were performed. The results obtained revealed promising antiproliferative effects in feline tumour cell lines, presenting different HER2 expression levels and with a conserved molecular mechanism of action, in comparison to a HBC cell line (SkBR-3). This mechanism induces a decrease in the phosphorylation levels of EGFR family members and its downstream pathways (Xia et al. 2002; Tanizaki et al. 2011; Formisano et al. 2014; Appert-Collin et al. 2015; Ma et al. 2017; Manning and Toker 2017; Matsumoto et al. 2018), and an increase in the phosphorylation of the suppressor tumour protein PTEN (R. Zhang et al. 2016). Particularly, considering lapatinib, this TKi presented the best antiproliferative effect in all the cells lines, revealing useful not only in HER2-positive, but also triple-negative feline mammary tumours, as described in human (Nielsen et al. 2004; Bouchalova et al. 2009). Furthermore, results demonstrated a stabilization and an increase in the non-phosphorylated membrane HER2, induced not only by lapatinib, as described (Scaltriti et al. 2009), but also by neratinib.

In parallel, immunotherapy with mAbs and ADC are widely used in HBC treatment protocols, and presents an alternative for TKi, which are known as toxic for the majority of tissues (Bonkobara 2015). Thus, by testing mAbs and ADC effects in the FMC cells lines, promising results were revealed, as an alternative for the treatment of diseased cats. *In vitro* assays, demonstrated that using mAbs (trastuzumab and pertuzumab) and the ADC, T-DM1 excellent antiproliferative effects were obtained, despite the need of a 3D system, that will allow a proper mAb-receptor interaction (Weigelt et al. 2010; Tatara et al. 2018). The cytotoxic effects revealed to be promising, not only in the HER2-positive (CAT-M and FMCm), but also in the HER2-negative (FMCp) cell lines, as suggested, e.g. the use of pertuzumab (Li et al. 2020) and T-DM1 (Endo et al. 2018; Nagayama et al. 2020). Furthermore, the mechanism of death induced by these compounds were conserved among feline and human cell lines, occurring by apoptosis (Cho et al. 2003; Richard et al. 2016; Yamashita-Kashima et al. 2017).

In this project, several combination protocols, which allow to block simultaneously different cell pathways were tested. This kind of therapeutic approach is recommended in HBC, increasing the effectiveness of the therapy, by the reduction of drugs' side effects, and surpassing the resistance development that occur along time (T. Liu et al. 2011; Tsang and Finn 2012; Tóth et al. 2016). The combined assays revealed important synergistic effects in the cell lines, among the drugs tested, namely by the combination of TKi (lapatinib and neratinib), with the mTOR inhibitor, rapamycin, which is a compound that *per se* did not induce a valuable cell cytotoxic response. In parallel, different combinations of mAbs (pertuzumab and trastuzumab) and mAbs plus the TKi, lapatinib were also tested with promising results, as well.

In breast cancer patients is known that innate or acquired mutations are a common mechanism of resistance to therapeutic drugs. These patients presented *her2* mutations in 2% to 3% of the HER2-positive mammary carcinomas and more than 70% occur in HER2-negative tumours (Mishra et al. 2017), as reported in this study for the cat. Analysing the feline *her2* gene sequence, namely the TK domain and the ECD (subdomain II and IV), the results obtained revealed similarities to what is described in the literature for women. Thus, *her2* mutations are more common in the TK domain, when comparing to the ECD (Gaibar et al. 2020). Moreover, intronic regions had the highest percentage of mutations, when compared to exons, being described as associated to cancer risk, tumour development, or therapy resistance in humans (Anczuków et al. 2012; Diederichs et al. 2016; Castagnoli et al. 2019; Høberg-Vetti et al. 2020). Furthermore, these type of mutations in the feline *her2* gene were already described as associated to the tumour development process, in cat (Santos et al. 2012).

For this research, the gene regions that codify for the protein locations recognized by the drugs tested were considered as the most important. Thus, a region of interest for the use of TKi is exon 20 in the *her2* gene, being mutations at this location, in human, associated to

an increase in the HER2 catalytic activity (Wang et al. 2006; Sun 2015). In parallel, in the feline tumour samples several mutations were found at exon 20, presenting a low frequency, and any of them described in human as induce resistance to therapy. Furthermore, analysing the TK domain of the *her2* gene, in the feline clinical samples, one mutation was found leading to the formation of a truncated form of the HER2 protein, which is not common in the ICD (Scott et al. 1993). These type of mutations occur more frequently in the ECD of the protein, leading to resistance to therapy. Additionally, in this region, one mutation was found as a prognostic marker, being associated with larger tumour sizes (c.19573 A>T; exon 18). Moreover, analysing the *her2* gene region that encodes for the protein ECD, most of the mutations were described in luminal B tumour subtype, while in women luminal B breast cancer mutations occur more frequently in *PIK3CA* and *TP53* genes (Ades et al. 2014). In the FMC clinical samples mutations revealed to be more common in exon 3, which encodes for the ECD, subdomain II in the HER2 protein, the location recognized by pertuzumab. Despite any of the mutations found in feline tumours are described in women as inducing therapy resistance, the results obtained suggest if resistance occur in cat, it will be more common to the use of pertuzumab.

Furthermore, in human is known that HER2 induces the expression of leptin in breast epithelial cells, presenting a close relation with breast cancer development (Cha et al. 2012). Taking this knowledge into account, and considering that, not only, the HER2-positive FMC is one of the most prevalent tumour subtype, with the HER2 identified as a prognosis biomarker (Maria Soares, Ribeiro, et al. 2016), but also being the obesity a common nutritional condition in cats, associated to an increase in leptin levels, the leptin/ObR axis was evaluated in diseased animals, in order to identify these proteins as tumour biomarkers. In human, this axis is associated to pro-inflammatory and pro-tumorigenic effects, being particularly relevant in obese post-menopausal women (Pan et al. 2018; Modzelewska et al. 2019). On the other hand, and as reported in pre-menopausal women with breast cancer (Georgiou et al. 2016) and patients with colon cancer (Arpaci et al. 2002), cats with mammary carcinoma presented a reduced FLI, which occurs due to a decrease in serum leptin levels, coupled with an increase in serum ObR levels (Chan et al. 2002; Garofalo et al. 2006; Hosney et al. 2017). Indeed, in this study, was demonstrated that higher leptin levels were correlated with ulcerated tumours and shorter DFS (Hosney et al. 2017; Modzelewska et al. 2019), and increased serum ObR levels being associated with small tumour sizes and an immunosuppressive status (Rivadeneira et al. 2019; Nascimento et al. 2020; Urbano et al. 2020), presenting a correlation with several immune checkpoint proteins, namely PD-1, PD-L1, CTLA-4 (Chen 2005; Erfani et al. 2010; Wang et al. 2016; Wei et al. 2016) and TNF-α (Montesano et al. 2005; Gautam Sethi, Bokyung Sung 2008; Singh et al. 2020). Thus, the results obtained suggest the use of these

proteins as diagnostic and prognosis biomarkers. Furthermore, its analysis as future adjuvant therapeutic targets could be revealed as a promising pharmacological alternative.

In sum, the obtained data along this project revealed promising new diagnostic/prognosis biomarkers, not only by the use of leptin and ObR molecules, as well as considering the mutational analysis of the *her2* gene. Furthermore, new therapeutic drugs were proposed for different FMC subtypes, targeting the HER2 protein, as well as adjuvant drugs to use in combined protocols, e.g. rapamycin, and leptin/ObR antagonists (Otvos et al. 2011; Guo et al. 2012; Surmacz 2013; Zabeau et al. 2014). Moreover, by the analysis to the *her2* gene, any evidence of therapy resistance to the drugs tested were revealed, corroborating the promising use of the HBC protocols, in diseased cats.

1.1. Future perspectives

A long way remains to walk, considering the main goal of a deep knowledge in FMC's development, therapeutic protocols and tumour biomarkers, in order to improve the clinical diagnosis, therapy options, and at the end cats' prognosis.

At this point, with the reported results about the *in vitro* cytotoxicity assays, using several drugs for HBC treatment, the next step suggested is to test the same drugs in *in vivo* assays, resorting to laboratory animal models, for proper cytotoxicity and drugs' toxicity assays, and then clinical trials in cats with mammary carcinomas, accurately diagnosed. Moreover, considering the promising results by the use of mAbs and an ADC tested in the FMC cell lines, it will be valuable the development of felinized mAbs that recognizes the HER2 protein, to test in *in vivo* feline models. Furthermore, and as reported by our group (Almeida et al. 2021) a large number of therapeutic drugs, with different targets could be revealed as valuable protocols for the treatment of feline mammary tumours, such as the HDACi and MTi, which is a field to explore.

Results presented in this project revealed the utility of the *her2* gene analysis as a prognostic factor and new diagnostic biomarkers, such as the leptin and its receptor. Although, several human tumour biomarkers could be investigated in cat, improving the FMC diagnosis and animal prognosis. Some studies are ongoing on my research group in order to identify different molecular biomarkers, such as the PD-1/PD-L1 (Nascimento et al. 2020), CTLA-4 and TNF-α (Urbano et al. 2020), VEGF (Nascimento et al. 2021), V-domain immunoglobulin suppressor of T cell activation (VISTA) (Gameiro, Nascimento, Correia, et al. 2021b) or lymphocyte activation gene 3 protein (LAG-3; ongoing research), but the list goes on, with a high number of possibilities to search. Thus, it will be interesting, in order to understand the FMC development and to improve the diagnosis/prognosis options and immune therapy targets, that this research continues for the identification of new tumour biomarkers. Moreover, it will be important to understand the effect of all the biomarkers identified in a clinical

population, resorting to prospective studies. Additionally, the use of anti-leptin (Gonzalez et al. 2009), anti-PD1 (Cimino-Mathews et al. 2016), or anti-VEGF (Sun et al. 2020), among other molecules, should be investigated as potential adjuvant therapeutic tools for the FMC.

For clinical proposes, not only a viable and fast diagnosis is important, but also the use of cheap materials and methods. Considering this, and as demonstrated with preliminary results, using blood/serum samples, it will be possible to develop a fast diagnostic *kit* for the identification of the HER2 expression, being the FMC HER2-positive, one of the most common and aggressive mammary tumours in cats, and the HER2 protein, already identified as a prognosis biomarker.

1.2. General conclusions

The cat is considered a promising *in vivo* oncology model (Zappulli et al. 2005), namely for spontaneous HER2-positive and triple-negative breast cancers (Maniscalco et al. 2013; Maria Soares, Madeira, et al. 2016). Although, efforts are needed to better understand the development mechanism and biology of FMC, in order to improve cats' prognosis and develop different therapeutic options, beyond the surgery. The research project described in this thesis was developed assuming the extensive knowledge in HBC, in order to find similarities among the two species and to improve the FMC diagnosis and immunochemotherapeutic options.

FMC tends to be diagnosed in a late stage, presenting ulcerated masses, or metastasis (Giménez et al. 2010) and the therapeutic alternatives available are scarce, being restricted to mastectomy (Michishita et al. 2016) and adjuvant therapeutic protocols with, however, limited success (Zappulli et al. 2005; McNeill et al. 2009; Giménez et al. 2010). This research prove that the use of TKi (lapatinib and neratinib) alone or in combination with the mTOR inhibitor, rapamycin, could become a valuable option for the treatment of FMC, with similar mechanisms of action when comparing to the knowledge in human cells. Additionally, combined protocols are beneficial, reducing drugs concentrations, which will imply a decrease in the side effects, and also a decrease in therapeutic resistance development, as described in humans, by blocking different cell pathways simultaneously. In parallel, mAbs (pertuzumab and trastuzumab) and an ADC compound (T-DM1), and also, combined protocols between mAbs and mAbs with the TKi, lapatinib were tested, revealing promising results, as well, and a common cell death mechanism, by apoptosis, when comparing to human cells response. An important aspect of the use of mAbs is the need to produce a proper felinized mAb to prevent an immune response in *in vivo* assays. Furthermore, through the analysis of the *her2* gene, from feline mammary carcinoma clinical samples, it has been possible to show that, despite several mutations were described in the TK domain and ECD of the gene, the studied population did not present any known mutations, described in women that lead to resistance

to therapy, according to National Cancer Institute, International Cancer Genome Consortium and COSMIC databases, assuming this occurrence as rare, in cat. In parallel the *her2* mutational analysis could revealed important prognosis factors, as described in this project, with one mutation found, associated to bigger tumours, a poor prognosis feature.

Moreover, the studies herein provide a rationale use for leptin/ObR as new biomarkers for the FMC, proving that diseased cats showed a decreased FLI, coupled with decreased serum leptin levels in animals with luminal B and triple-negative subtypes. On the other hand, an increase in serum ObR levels was detected in all FMC subtypes, being associated to an immunosuppressive status. Additionally, the data reported suggests that cats with luminal B or triple-negative subtypes could benefit from adjuvant therapy targeting leptin/ObR. Furthermore, similarities between the feline and human tumour micro- and serological environments have also been revealed, suggesting equivalent tumour diagnostic and prognostic biomarkers, as well as the possible use of adjuvant treatments recommended in breast cancer therapeutic protocols (Rene Gonzalez et al. 2009; Cimino-Mathews et al. 2016; Nascimento et al. 2020). Thus, the information revealed in this task introduces a new research line for the understanding of the FMC development and adjuvant therapeutic alternatives, e.g. the use of anti-leptin (Gonzalez et al. 2009), anti-PD1 (Cimino-Mathews et al. 2016), or anti-VEGF (Sun et al. 2020) molecules.

This project was an important beginning in the therapeutic filed, since no specific therapy exists for the treatment of FMC. The known therapeutic options and protocols for the HBC are many, providing good results, improving patients DFS and OS times. Taking advantage of this knowledge, different therapeutic targets, drugs and adjuvant protocols could be considered and investigated for the diseased cats, in order to provide quality of life and improve the DFS and OS. To conclude, forthcoming perspectives include a deeper knowledge of FMC, defining proper diagnostic and prognostic biomarkers that can be used in clinical practice, and improve the therapeutic options for cats.

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Annexes

Supplementary Data

Annex I

Chapter II – Supplementary data

Table S1.1. Immunohistochemic characterization of the FMC cell lines (CAT-M, FMCp and FMCm), and the human breast cancer cell line (SkBR-3).

Cell Line	ER*	PR**	Ck5/6	Ki-67 (%)
CAT-M	3	8	Negative	50.2
FMCp	7	0		57.4
FMCm	3	0		68.5
SkBR-3	0	4		53.6

*ER – Oestrogen receptor; **PR – Progesterone receptor.

Table S1.2. Analysis of the *her2* TK domain mutations in the feline mammary carcinoma clinical samples. DNA samples were acquired from tumour mammary tissues, and PCR and sequencing steps were performed with specific primers.

Nucleotide Position	Mutations Frequency	Breed[1] (n)	Age Class[2] (n)	Size Class[3] (n)	Tumour Subtype (n)	Stage (n)	mRNA (bp); aa Modification	Observations*
19572 C > A	3	Ind	2 (2); 3	2 (3); 3	LB (2); TN	3 (2); 4	2153; T > K	Substitution (Polar neutral to polar basic)
19573 A > C	4	Ind (3); SM	2	2; 3 (3)	LB (3); TN	2; 3 (3)	2154; T	Synonymous
19575 G > T	1	Ind	2	2	LB	2	2156; E > D	Substitution (Polar neutral to polar acid)
19581 G > T	2	Ind	2	2; 3	LB	2; 3	2162; R > M	Substitution (Polar basic to apolar)
19584 A > G	3	SM	2 (2); 3	2 (2); 3	TN; LB-HER2; LB	2; 3 (2)	2165; K > E	Deletion AAG to -AG
19584 / 19585 AG > CC	1	Ind	2	2	LB	2	2165 / 2166; K > T	Substitution (Polar basic to polar neutral)
19587 T > G	1	Ind	2	2	LB	2	2168; V > G	Substitution (both apolar)
19593 T > C	1	Ind	2	2	LB	2	2174; V > A	Substitution (both apolar)
19604 G > T	1	SM	2	3	LB	3	2185; G > STOP	Substitution
19607 G > A	1	Ind	2	3	LB	3	2188; A > T	Substitution (Apolar to polar neutral)
19626 Del	1	Ind	2	3	TN	3	2207; K > R	Deletion AAG to A-G
19631 A > T	1	Ind	2	2	LB	2	--	Intron
19643 A > C	2	Ind (2)	2 (2)	2;3	LB (2)	2; 3	--	Intron
19666 A > T	1	Ind	2	2	LB	2	--	Intron
19667 G > T	1	Ind	2	2	LB	2	--	Intron – Homozygous sample Increased mRNA levels (n= 1)[#]

19676 G > A	3	Ind (3); SM	2 (3)	2 (2); 3	LB (2); TN	2; 3; 4	--	Intron
19677 T > C	3	Ind (3)	2 (2); 3	2 (3)	HER2; LB (2)	2 (2); 3	--	Intron
19712 A > C	2	Ind (2)	2 (2)	2; 3	LB (2)	2; 3	--	Intron
19713 A > T	2	Ind (2)	2 (2)	2; 3	LB (2)	2; 3	--	Intron
19714 G > A	4	Ind; SM	2 (2)	2; 3	LB-HER2; LB	3 (2)	--	Intron
19716 G > C	1	Ind	2	3	LB	3	--	Intron
19822 G > A	1	NF	2	2	LB	3	--	Intron
19828 G > C	1	Ind	2	2	LB	2	--	Intron
19845 G > T	1	Ind	2	2	LB	2	--	Intron
19858 G > T	1	Ind	2	2	LB	2	--	Intron
20044 G > A	2	Ind (2)	1; 2	3; 1	HER2; LB-HER2	3; 1	--	Intron
20045 A > G	2	Ind	2	3	HER2	3	--	Intron
20142 G > A	17	Ind	1	1	LB-HER2	1	--	Intron Increased mRNA levels (n=2) [#]
20278 T > C	5	Ind (5)	2 (2); 3 (3)	1; 2 (3); 3	LB (5)	1; 3 (3); 4	--	Intron
20289 G > A	12	Ind (9); SM (2); Per	2 (5); 3 (7)	1 (2); 2 (8); 3 (2)	LB (5); LB-HER2 (2); TN (3); HER2 (2)	1 (3); 2 (4); 3 (5)	--	Intron
20380 C > G	5	SM (3); Per; Ind	2 (3); 3 (2)	1; 2 (3); 3	LB (2); LB-HER2; TN (2)	1; 2; 3 (3)	2404; P > A	Substitution (both polar neutral)
20382 T > C	1	Per	3	1	TN	1	2406; P	Homozygous; Synonymous
20384 A > T	1	Per	3	1	TN	1	2408; Y > F	Substitution (Polar neutral to apolar)
20385 T > G	2	Ind	3; 2	1; 2	LB; LB-HER2	1; 2	2409; Y > STOP	Substitution
20428 G > C	2	Ind	1; 3	1 (2)	LB-HER2	1	2452; G > R	Substitution (Apolar to polar basic)
20436 G > A	1	Ind	1	1	LB-HER2	1	2460; E	Synonymous

20459 A > T	1	Ind	1	1	LB-HER2	1	2483; E > L	Substitution (Polar neutral to apolar)
20531 C > T	4	Ind; SM; Per (2)	2 (3); 3	1; 2; 3 (2)	TN (2); LB (2)	1; 3 (2); 4	--	Intron Increased mRNA levels (n=1) [#]
20533 G > A	2	Ind (2)	2; 3	1; 3	HER2; LB-HER2	1; 3	--	Intron
20612 G > A	1	Ind	2	3	HER2	3	--	Intron
20827 A > G	7	Ind (7)	2 (3); 3 (4)	1; 2 (4); 3 (2)	LB (7)	1; 3 (5); 4	--	Intron Equal mRNA levels (n=3) [#]; Increased mRNA levels (n=4) [#]; Decreased mRNA levels (n= 1) [#]
20940 T > G	34	Ind (28); SM (4); Per (2); NF	1 (2); 2 (18); 3 (14)	1 (8); 2 (17); 3 (9)	LB (17); LB-HER2 (8); TN (7); HER2 (2)	1 (8); 2 (5); 3 (18); 4 (3)	2716; W > G	Substitution (both apolar)

[1]Ind – Indeterminate; SM – Siamese; NF – Norwegian Forest Cat; [2]Age class 1< 8 years; 2 from 8 to 12 years; 3> 12 years; [3]Size class 1< 2 cm; 2 from 2 to 3 cm; 3> 3 cm; *All the mutations not specified were heterozygous; [#] Reported by Ferreira et al. 2019 (Ferreira, Soares, et al. 2019).

Table S1.3. Feline cell lines presented a few number of mutations in the feline *her2* TK domain, and none of them were described in women, as leading to TKi resistance. DNA samples were acquired from cells in culture, and PCR and sequencing steps were performed with specific primers.

	CAT-M			
	18/19	**19/20**	**20/21**	**21/22**
Intron (bp)	19677 T > C	20142 G > A	--	20827 A > G

	FMCp						
	18/19	**19/20**	**20/21**	**21/22**		**20***	**21**
	19677 T > C	20063 T > G	20560 A > G	20804 A > G		20312 G > A	20657 G > C (Arg to Gly)
		20077 G > T	20584 T > C	20816 T > C		20327 C > A	20684 C > T (Arg to Cys)
		20091 A > G		20822 C > A		20339 A > G	20716 C > G (Leu to Val)
		20105 G > A		20831 C > T		20348 T > C	
		20136 C > G		20857 C > A		20354 T > C	
Intron (bp)		20141 C > T			**Exon (bp)**	20366 T > C	
		20175 A > C				20405 T > C	
		20183 G > A				20408 T > C	
		20218 G > T				20450 A > G	
		20242 G > C				20436 C > T	
		20257 T > C					

	FMCm			
	18/19	**19/20**	**20/21**	**21/22**
Intron (bp)	19677 T > C	20142 G > A	--	20827 A > G

*Synonymous mutations.

Annex II

Chapter III – Supplementary data

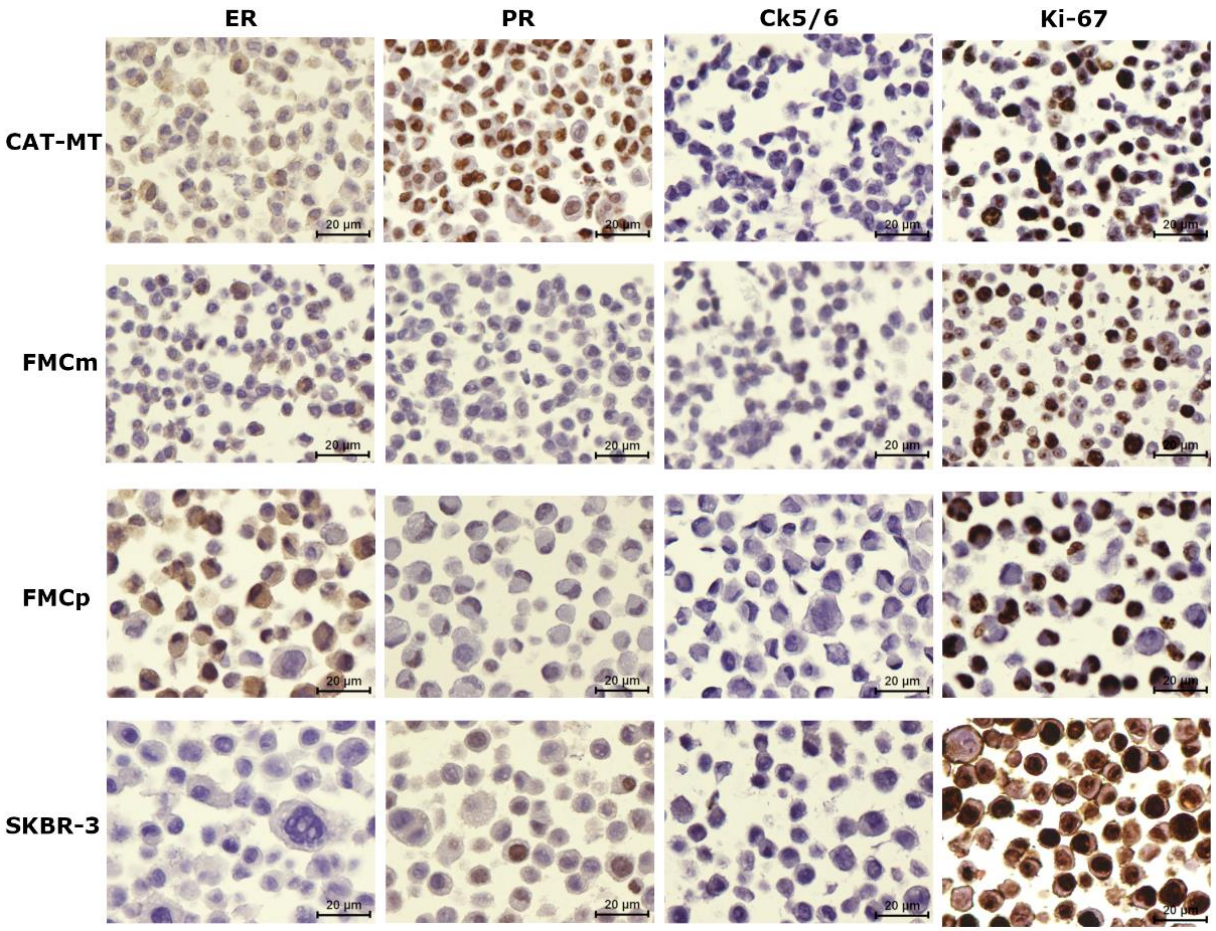

Figure S2.1. Characterization of the FMC cell lines (CAT-MT, FMCm and FMCp) and the human SkBR-3 cell line, by immunocytochemistry. (400x magnification)

Table S2.1. Summary of the characterization of the FMC (CAT-MT, FMCm and FMCp) and human breast cancer (SkBR-3) cell lines, and its origin.

Cell Line	Tumour origin	ER*	PR**	Ck5/6	Ki-67 (%)
CAT-MT	Mammary adenocarcinoma	3	8	Negative	50.2
FMCm	Metastatic lymph node	3	0		68.5
FMCp	Primary breast tumour	7	0		57.4
SkBR-3	Mammary adenocarcinoma	0	4		53.6

*ER – Oestrogen receptor; **PR – Progesterone receptor

Table S2.2. Analysis of *her2* ECD mutations, subdomains II and IV, which encodes for the regions recognized by pertuzumab and trastuzumab, respectively, in the feline mammary carcinoma clinical samples.

Region	Exon	Nucleotide Position (bp)	Mutations frequency	Breed[1] (n)	Age Class[2] (n)	Size Class[3] (n)	Tumour subtype (n)	Stage (n)	mRNA (bp); aa modification	Observations*
II	3	7554	2	Ind (2)	2; 3	2 (2)	LB (2)	2; 3	231; Ile	Synonymous; Apolar
		7563	2	Ind (2)	2 (2)	1; 2	LB; LB-HER2	1; 2	240; Val	Synonymous; Apolar
		7568	4	Ind (3)	2 (2); 3	1 (2); 2 (2)	LB (3); LB-HER2	1 (2); 2; 3	245; Gly to Val	Apolar
		7569	1	Ind	2	2	LB-HER2	3	246; Gly	Synonymous; Apolar
		7572	2	Ind; SM	2 (2)	2; 3	LB (2)	2; 3	249; Tyr	Synonymous; Polar neutral
		7579	1	Ind	2	2	LB	2	256; Ile to Leu	Apolar
		7587	1	Ind	3	2	LB	3	246; His to Gln	Polar basic to Polar neutral
		7595	1	Ind	2	2	LB	2	272; Val to Glu	Apolar to Polar acid
		7609	1	Ind	2	2	LB-HER2	3	286; Leu	Synonymous; Apolar
		7618	5	Ind (5)	2 (4); 3	1; 2 (2); 3 (2)	LB (4); LB-HER2	1; 3 (3); 4	295; Leu to Val	Apolar
		7626	1	Ind	3	2	LB	3	303; Ile to Met	Apolar
		7644	4	Ind (4)	2 (4)	2 (2); 3 (2)	LB (3); LB-HER2	2 (2); 3; 4	321; Leu	Synonymous; Apolar
		7656	6	Ind (4); SM; NF	2 (6)	1; 2 (2); 3 (3)	LB (5); LB-HER2	1; 2 (2); 3 (2); 4	333; Asn to Lys	Polar neutral to Polar basic
		7668	1	Ind	2	2	LB	3	345; Ala	Synonymous; Apolar
		7672	3	Ind (2); NF	2 (3)	1; 2; 3	LB (3)	1; 2; 3	349; Leu	Synonymous; Apolar
		7674	1	Ind	2	2	LB-HER2	3	351; Leu	Synonymous; Apolar
		7690	1	Ind	2	2	LB	2	367; Leu / Leu to Val	Heterozygous; Synonymous / or change de codon; Apolar
		7696	1	Ind	3	2	LB	3	373; Asn to Thr	Polar neutral

		7706	1	Ind	2	1	LB	1	383; Pro to His	Apolar to Polar basic
		7711	1	Ind	2	2	LB	3	388; Thr to Ala	Polar neutral to Apolar
		7718	2	Ind (2)	2 (2)	1; 2	LB; LB-HER2	1; 2	395; Ala to Gly	Apolar
		7727	1	Ind	2	2	LB	3	404; Gly to Ala	Apolar
		7735	1	NF	2	3	LB	3	412; Arg	Synonymous; Polar basic
		7741	4	Ind (3); SM	2 (4)	1; 2 (2); 3	LB (2); LB-HER2 (2)	1; 2; 3 (2)	418; Leu to Val	Apolar
		7756	3	Ind (2); NF	2 (3)	1; 2; 3	LB (3)	1; 3; 4	433; Leu to Phe	Heterozygous; Apolar
	4	No mutations reported in the sequenced regions, from c.8575bp to c.8709bp								
	10	13320	2	Ind; Per	2 (2)	2 (2)	LB; TN	4 (2)	1193; Arg to Ile	Heterozygous; Polar basic to Apolar
		13435	1	Ind	2	2	TN	4	1233; Tyr	Heterozygous, synonymous; Polar neutral
		13451	2	Ind (2)	2 (2)	2 (2)	LB; TN	3; 4	1249; His to Asn	Heterozygous; Polar basic to Polar neutral
	11	13458	1	Ind	2	2	TN	4	1256; Leu to Ser	Heterozygous; Apolar to Polar neutral
		13464	1	Ind	2	2	TN	4	1262; Leu to Ser	Apolar to Polar neutral
IV		13485	1	Ind	2	2	TN	4	1283; Leu to His	Apolar to Polar basic
	12	13772	1	Ind	2	3	LB-HER2	3	1365; Leu	Synonymous; Apolar
		13917	1	Ind	1	1	LB-HER2	1	1510; Cys to Gly	Polar neutral to Apolar
		14299	1	Ind	2	1	LB	1	1536; Tyr	Synonymous; Polar neutral
	13	14403	1	Ind	3	1	TN	1	1640; Leu to Arg	Apolar to Polar basic
		14406	1	Ind	2	1	LB	1	1643; Gln to Pro	Base insertion; Polar neutral to Apolar

[1]Ind – Indeterminate; SM – Siamese; NF – Norwegian Forest Cat; [2]Age class 1< 8 years; 2 from 8 to 12 years; 3> 12 years; [3]Size class 1< 2 cm; 2 from 2 to 3 cm; 3> 3 cm; *All the mutations not specified were homozygous.

Table S2.3. Feline cell lines presented a small number of mutations in the feline *her2* ECD, subdomains II (exons 3 and 4) and IV (exons 10 to 13), with none of them reported in human as leading to resistance to therapy. DNA samples were acquired from cells in culture, and PCR and sequencing were performed with specific primers.

Subdomain	Exon	Cell Line	gDNA mutation (bp)	Protein change
II	3	CAT-MT	c.7684 G>A	Asp to Asn Polar acid to polar neutral
		FMCm	c.7622 G>A	Arg to Gln Polar basic to apolar
		FMCp	c.7554 C>A	Ile Apolar (Synonymous)
			c.7568 G>T	Gly to Val Apolar
			c.7572 / c.7573 TG>CC	Tyr / Val to Leu Polar neutral to apolar
			c.7644 C>A	Leu Apolar (Synonymous)
			c.7647 T>A	Phe to Leu Apolar
			c.7656 C>G	Asn to Lys Polar neutral to polar basic
			c.7668 C>A	Ala Apolar (Synonymous)
			c.7672 C>T	Leu Apolar (Synonymous)
			c.7756 C>T	Leu to Phe Apolar
	4	No mutations reported		
IV	10	FMCp	c.13303 G>A	Ala to Thr Apolar to polar neutral
			c.13345 T>C	Ile Apolar (Synonymous)
	11	FMCp	c.13470 T>C	Leu to Ser Apolar to polar neutral
			c.13514 C>A	Ala Apolar (Synonymous)
	12	FMCm	c.13836 G>A	Val to Ile Apolar (Heterozygous)
	13	No mutations reported		